ISOLATION AND CHARACTERIZATION OF COMPONENTS FROM WHEY

BY

YUE XU

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Supervisory Panel
Assoc. Prof. Jim Hourigan, University of Western Sydney, School of Food Science
Dr. Robert Sleigh, Australian CSIRO, Division of Food Science and Technology
Dr. Bob Johnson, Australian CSIRO, Division of Food Science and Technology
PLEASE NOTE

The greatest amount of care has been taken while scanning the following pages. The best possible results have been obtained.
The candidate, Yue Xu, hereby declares that this submission is his own work except where due acknowledgment is made in the text, and that none of the work in this thesis has been submitted to any other university or institution for a higher degree.

Yue Xu
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SYNOPSIS

The structure, functionality, isolation methods and applications of whey components, particularly the proteins and lactose, have been extensively studied. These studies have had a great impact on the food industry where whey components are increasingly being used as food ingredients. Two generations of whey protein product, namely Lactalbumin, produced by heat-induced precipitation, and Whey Protein Concentration / Isolate, produced by ultrafiltration / ion exchange chromatography, have been commercialised. Crystalline lactose in the food and pharmaceutical grades is also being produced. Recently, research activities in whey fractionation have shifted to the isolation of the minor components, such as β-lactoglobulin, lactoferrin, glycomacropeptide and immunoglobulins, and to reduce the disposal of whey or permeate as wastewater.

Many technologies have been and are being developed for the isolation of whey components. However, the balance between the portion of whey components recovered and the disposal of whey in the wastewater stream has not changed. This thesis is aimed at developing a Total Whey Utilization strategy by which the several components of the whey stream would be completely recovered by fractionation, resulting in little or no residue to be disposed of in the wastewater stream. Therefore, this study was initially dedicated to the development of novel separation methods which would be suitable for the Total Whey Utilization process. The development of those techniques revealed some previously unknown features of whey components. The mechanisms of the separation methods have been also investigated.
Whey has always been difficult to treat by ion exchange and membrane processes since they have variable composition and contain suspended matters. To minimise these problems various pretreatments, e.g. calcium chloride addition to precipitate the suspension from Cheddar cheese whey was developed in the 1980s. But little is known about the constituents of the precipitate. In this work, an investigation of the composition of the precipitate obtained from both Cottage and Cheddar cheese whey by addition of calcium and phosphate at pH 7.5 has shown that most of the Lactoferrin found in whey co-precipitates with calcium phosphate precipitation. This was verified by an “in vitro” experiment with model solutions in which standard lactoferrin co-precipitated with calcium phosphate at pH 7.5. This discovery not previously reported has led to the development of a Pretreatment, which involves addition of calcium and phosphate ion, adjustment of pH to 7.5, then centrifugation, to recover about 50-70% of lactoferrin from Cottage and Cheddar cheese whey. Simultaneously the pretreatment clarifies the wheys. Highly purified lactoferrin can be obtained by extraction of the precipitate followed by ion exchange chromatography. The capital and operation cost of producing lactoferrin by this process should be less than other processes since lactoferrin has been pre-concentrated in the pretreatment step and a much smaller chromatographic unit will be used.

Examination of the interactions of bovine lactoferrin with Ca\(^{2+}\) ion and other cations by Fourier transform infrared spectroscopy (FTIR), fluorimetry and spectrophotometry shows that calcium complexes with lactoferrin by binding to the carboxylic side chain and suggests that precipitation of lactoferrin from whey is due to complexation of the protein by calcium. This Ca\(^{2+}\)-binding feature when coupled with the result of the “in vitro” experiments led to the proposal in this study that lactoferrin
is precipitated as a Lactoferrin-Ca\(^{2+}\)-PO\(_4\) complex in which the calcium ion is a ligand binding to the protein. The "pseudo-affinity precipitation" model developed in this work to explain the co-precipitation of lactoferrin with calcium phosphate is supported by the report of Bennet et al. in 1981 (see Chapter 4) that human lactoferrin forms a tetramer in the presence of calcium.

Calcium binding also affects the secondary structure of bovine lactoferrin. It was observed that in the presence of calcium ion as much as 27-46% of the \(\alpha\)-helix was reduced, whereas there was an increase of 19-23% in \(\beta\)-structure. This calcium-ion induced change of the secondary structure may explain the previous reports (see Chapter 4) that calcium ions reduced or abolished the antimicrobial activity of lactoferrin.

The separation of glycomacropeptide and immunoglobulins from whey was also investigated. A chromatographic process was developed to selectively adsorb glycomacropeptide from Cheddar cheese whey. Immunoglobulins were isolated from whey by ion exchange and membrane techniques through the removal of the major protein impurities such as \(\alpha\)-lactalbumin, \(\beta\)-lactoglobulin, and bovine serum albumin. Enzyme-Linked Immunosorbent Assays was used to monitor the immunoglobulin contents of the fractions.

Lactose is usually produced from permeate by crystallization. Although crystallization is an efficient method for fractionation or purification, its disadvantage is that the mother liquor is a wastewater containing high salt and BOD. The chromatographic method has been investigated in this work to separate the mother liquor or permeate into lactose and mineral fractions such that a goal of this thesis, namely a "clean" water stream after processing whey, can be finally achieved. These
studies have focused on the effect of resin type, salt form of the resin and the operating conditions on the separation of the lactose and mineral fraction.

* In this study, "in vitro" has been used to designate the precipitation experiments by using standard lactoferrin.
# ABBREVIATIONS MOST IN USE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>BLf or bLf</td>
<td>Bovine Lactoferrin</td>
</tr>
<tr>
<td>BOD</td>
<td>Biological Oxygen Demand</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDP</td>
<td>Casein-derived Peptides</td>
</tr>
<tr>
<td>CM</td>
<td>Carboxymethyl</td>
</tr>
<tr>
<td>CN</td>
<td>Casein</td>
</tr>
<tr>
<td>DF</td>
<td>Diafiltration</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DVB</td>
<td>Divinylbenzene</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GMP</td>
<td>Glycomacropeptide</td>
</tr>
<tr>
<td>HLF or hLF</td>
<td>Human Lactoferrin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>Igs</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-Dalton</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Distribution Coefficient</td>
</tr>
<tr>
<td>La</td>
<td>α-Lactalbumin</td>
</tr>
<tr>
<td>Lg</td>
<td>β-Lactoglobulin</td>
</tr>
<tr>
<td>Lp</td>
<td>Lactoperoxidase</td>
</tr>
<tr>
<td>Lpn</td>
<td>Lactophorin</td>
</tr>
<tr>
<td>Ly</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>MFGM</td>
<td>Milk Fat Globule Membrane</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-schiff</td>
</tr>
<tr>
<td>Pi</td>
<td>Phosphate</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PP</td>
<td>Proteose-Peptone</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate — Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethyl ethylene diamine</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2(hydroxy-methyl) propane-1,3 diol</td>
</tr>
<tr>
<td>TWP</td>
<td>Total Whey Protein</td>
</tr>
<tr>
<td>UF</td>
<td>Ultrafiltration</td>
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<tr>
<td>------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>D</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
</tr>
<tr>
<td>Threonine</td>
<td>T</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>E</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
</tr>
<tr>
<td>Proline</td>
<td>P</td>
</tr>
<tr>
<td>Glycine</td>
<td>G</td>
</tr>
<tr>
<td>Alanine</td>
<td>A</td>
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<tr>
<td>Cysteine</td>
<td>C</td>
</tr>
<tr>
<td>Valine</td>
<td>V</td>
</tr>
<tr>
<td>Methionine</td>
<td>M</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>I</td>
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<tr>
<td>Leucine</td>
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<tr>
<td>Tyrosine</td>
<td>Y</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>W</td>
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FORMATS

This thesis has been prepared according to a manuscript plan in which each chapter is a “stand alone” manuscript dealing with a discrete aspect of the overall study. Chapter 1 and 2 set the scene and place for the work in the context of the literature while the later chapters focus on more defined experimental aspects.

Chapters 1 and 2 have been written according to the format of Food Reviews International.

Chapters 3, 4, 5 and 6 have been written according to the format of International Dairy Journal.
Chapter 1 Literature Review 1: Structure, Functionality, Isolation and Utilization of Whey Proteins and Lactose

Abstract

The structure, functionalities, isolation methods and utilization of whey proteins and lactose, are extensively reviewed in this chapter. Nearly all the known proteins and peptides of whey, notably α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulins, lactoferrin, enzymes and casein-derived peptides, are discussed in this chapter. Strategies proposed for developing new or modified isolation methods are studied in more detail in the following chapters.
1.1 Introduction

Whey is defined [1] as the fluid obtained by coagulating and separating the casein proteins from whole or skim milk. Sweet whey results from using rennet-type enzymes at a minimum pH of 5.6 to induce the coagulum; acid whey is produced from forming the coagulum by acidifying the milk to a maximum pH of 5.1 with either a lactobacillus culture or mineral acid. Whey is a dilute liquid containing approximately 6% total solids of which 70% or more is lactose and about 9% whey proteins. The typical compositions of different wheys are given in Table 1-1.

In 1986 the world production of whey was estimated to be 102-110 million tonnes [3, 4] of which U.S.A. share was approximately 20 million tonnes. A 1991 survey of Australian industry gave an estimate of 1,560 million litres of whey produced each year [5]. Only 50% of this whey is utilised in the manufacture of whey powder, lactose and whey protein concentrate. The remaining 50% is disposed of, mostly by spraying onto land, some as animal feed and a small amount goes into the sewage system and ocean outfalls.

Whey has a very high biological oxygen demand (BOD) of 35,000-45,000 mg/litre [6] and its disposal is a major environmental problem for the dairy industry: disposal into streams and onto land are no longer satisfactory solutions. Lactose and protein represent about 70% of the whey BOD [7], so the problem of whey disposal can be eased by recovery of these components; their sale would be an economic benefit to the industry. Only 50% of the world’s annual whey production is used as whey powder, delactosed whey powder, lactose and whey protein concentrate, but
the increasing demand for whey products and lactose can be anticipated by current trends in the whey trade (Table 1-2).

This review is aimed at:
i) Discussing the state of current knowledge of the structures, functionalities, isolations and utilizations of the two major components of whey, i.e., protein and lactose.

ii) Providing a foundation on which a strategy can be proposed for achieving “Total Whey Utilization”.

1.2 Structures of Whey Proteins and Lactose

1.2.1 Structures of Whey Proteins

As a food ingredient whey protein is valued for its high nutritional quality, and is used in this role. However, the nutritional use does not realise the full value of whey protein. Whey protein is made up of many individual proteins and peptides some of which have valuable physical properties, e.g. whippability, and many have value for their biological functions such as antimicrobial properties.

The distribution and notable properties of some typical whey proteins are listed in Table 1-3. Compared with the casein proteins, which are responsible for the "coagulum", whey proteins are more heat-sensitive, less calcium-sensitive, and can engage in thiol-disulfide interchanges to form oligomeric structures [10].

In the following, the proteins and peptides of whey, including α-lactalbumin (La), β-lactoglobulin (Lg), bovine serum albumin (BSA), casein-derived peptides
(CDP), lactoferrin (Lf), lactoperoxidase (Lp) and immunoglobulins (Igs) are reviewed with particular attention being paid to the bioactive proteins and peptides such as CDP, Lf, Lp and Igs, with respect to their structures, functionalities, potential uses and isolations.

1.2.1.1 α-Lactalbumin

The major whey proteins are referred to α-lactalbumin, β-lactoglobulin and bovine serum albumin. They account for up to 80% of the total whey protein (Table 1-3), and have been extensively characterized [10,12,13,14].

La accounts for 5-25% of whey protein. The ratio of La to Lg in whey is approximately 1:3. La is a compact globular protein (14,000 Da) and its complete amino acid sequences from 10 mammalian species have been determined by direct protein sequencing or translation from cDNA or a combination of the two approaches [15].

From what is known about its three-dimensional structure, La has a compact globular structure (14,000 Da) containing four disulfide bonds, four α-helices, two regions of β-structure, several regions of consisting of a helix containing 3 turns per 10 residues (i.e. a 3_10 helix), and a Ca^{2+}-binding loop. In this loop Ca^{2+} is surrounded by seven ligands, all oxygens, three from the carboxylate groups of Asp (residues 82, 87 and 88), two carbonyl oxygen atoms (from residues 79 and 84) and two water molecules [16]. Table 1-4 gives details about the three-dimensional structure of La.

1.2.1.2. β-Lactoglobulin
β-Lactoglobulin is the most extensively characterized and best described of all food proteins. The amino acid sequence of Lg has been well established [17]. The protein exits as a dimer in solution because of electrostatic interactions between Asp130 and Glu134 of one monomer with corresponding lysyl residues of another monomer. The native conformation is sensitive to heat and pH; at temperatures below 25°C and pH values above 7.0, the protein forms octamers. Native Lg possesses two disulfide bonds (Cys66-Cys160 and Cys106-Cys119), and a free thiol group (Cys121) which is inaccessible to solvent at or below neutral pH [18]. The secondary structure of Lg has been calculated from circular dichroism and infrared spectroscopy data and it contains approximately 15% α-helix, 51% β-sheet, 17% turns, and 17% aperiodic structure [17]. Recently, the three-dimensional structure of Lg was determined by high-resolution X-ray crystallography (2.8 Å) [18].

1.2.1.3. Bovine Serum Albumin

BSA in whey is a large globular protein (66,000 Da) with a good essential amino acid profile. It consists of a single polypeptide chain containing about 580 amino acid residues with 17 intrachain disulfide bonds and one free thiol group at residue 34. Although the precise three-dimensional crystal structure of BSA is not known, the distribution of disulfide bonds and the location of specific residues throughout the polypeptide chain suggest that the albumin molecule folds to form three structural domains and nine subdomains [19].

1.2.1.4. Casein-derived Peptides

The casein-derived peptides of whey are a heterogenous group formed by proteolysis of the caseins by both endogenous and exogenous proteinases. Alkaline
milk protease is an example of a major endogenous proteinase of bovine milk. This enzyme is thought to be plasmin [20, 21], an enzyme of bovine serum responsible for catalyzing the dissolution of fibrin clots. Rennin is an example of an exogenous proteinase. It is added to milk to induce the curd for cheesemaking.

When most of the proteins including caseins and whey proteins are coagulated by heating milk to 95°C and adjusting the pH to 4.6, proteose-peptone (PP), a heat-stable and acid-soluble fraction, can be separated from the whey [22]. The PP fraction is actually a heterogeneous group of phosphoglycoproteins formed following proteolysis of the N-terminal region in the sequence of β-casein (β-CN) by plasmin [10]. The PP consists of 4 electrophoretically different fractions. Three of them, namely component 5 (C-5) identical with β-CN residues 1-105 or 1-107, component 8-fast (C-8-fast) with β-CN residues 1-28 and component 8-slow (C-8-slow) with β-CN residues 29-105 or 29-107 (Table 1-3), are known to be peptides derived from β-casein (β-CN) digested by endogenous plasmin [9]. Another fraction, component 3, contains several glycoproteins, some of which have similar antigenicity to the glycoproteins of milk fat globule membrane (MFGM) [23]. Because bovine milk plasmin is associated with both casein and MFGM fraction [9], it is possible that fragments of MFGM polypeptides digested by plasmin are found in component 3. One of the major glycoproteins in the proteose-peptone component-3 fraction, named as lactophorin (LPn), was purified [24]. Recently cDNA of LPn was cloned [25]. LPn is composed of two main glycoproteins (27 and 17 kDa).

In the manufacture of rennet casein or Cheddar and similar cheeses, the curd results from the action of exogenous rennet-type enzymes on κ-casein. Rennin
(chymosin) cleaves bovine κ-casein at the $^{105}$Phe$^{106}$Met bond (Fig.1-1) into para-casein and the κ-casein glycomacropeptide (GMP) [12]. The GMP is a highly acidic and hydrophilic peptide with a molecular weight of 7,000 Daltons (Table1-3) suggested by both the theoretical calculation from hydrolysis of the κ-casein molecule at the 105-106 peptide bond and the experimental value determined by Sephadex G-100 gel filtration in pH 8.6, 7 M urea buffer. The work conducted by Morr and Seo [26], however, reported one major GMP peak with a molecular weight of 33,000 Daltons determined by size exclusion high pressure liquid chromatography (HPLC) and a group of size-heterogenous peptides with molecular weights of ~18,000 daltons revealed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Milk contains a number of antimicrobial components for the protection of the newborn animal. These components are usually present in very low concentrations in milk, and most remain in the whey fraction when the casein is precipitated by the action of acid or rennet. Some important anti-microbially active proteins of whey are listed in Table 1-5. The antimicrobial or protective proteins in milk can be divided into two groups; the immunoglobulins (Igs) and non-immunoglobulins.

1.2.1.5. Immunoglobulins

Igs, also well known as antibodies, belong to a family of related structural glycoproteins. There are five major isotypes: IgG, IgA, IgD, IgE and IgM. These isotypes differ from each other structurally with respect to size, electrophoretic charge, and carbohydrate content [28]. Of these IgA, IgG and IgM are found in bovine milk [29]. Nielsen [30] claimed IgE also existed in bovine milk. IgG
together are the predominant bovine Igs, accounting for about 80% of the total (Table 1-5). IgG is further divided into two subclasses, IgG1 and IgG2 according to antigenicity and electrophoretic mobility. The basic structure of IgG as a representative, is shown in Fig.1-2. It is composed of two identical light chains (23,000 dalton) and two identical heavy chains (55,000 Dalton). The four chains are joined together with disulfide bonds. The complete molecule has a molecular weight of approximately 150,000 Dalton. The two identical antigen binding sites are each made of the N-terminal part of one heavy and one light chain. The basic structure of monomeric IgA and IgM is similar to that of IgG except for the addition of a C-terminal octapeptide to the heavy chains. The other difference is that IgA and IgM occur as dimers and pentamers, respectively, thus they have greater apparent molecular weights than IgG. The concentrations of Igs in the milk whey accounted for only 5-6% of total whey protein as compared with 77% of total whey protein in the colostral whey (Table 1-3).

Lactoperoxidase (Lp), lactoferrin (Lf) and lysozyme (Ly) (Table 1-5) comprise the non-immunoglobulin protective system in milk. These three components appear in the milk of most species and have been well characterised. The concentrations of Lf and Ly in bovine milk are 0.02-0.35 mg/ml and 0.1-0.2 μg/ml (Table 1-5), respectively, much lower than those of human milk, 1.5 mg/ml and 100 μ g/ml, respectively [31]. Human milk, however, lacks Lp [32].

1.2.1.6. Lactoperoxidase and Lysozyme (Ly)

Lactoperoxidase is a glycoprotein with a molecular weight of ~ 78 kDa that contains one heme group. The iron content is 0.0680-0.0709 % and the carbohydrate
content 9.9-10.2% [33]. The primary structure of Lp is unknown but information on the secondary structure indicated that 65% of the molecule exists as β-structures, 23% as α-helix and 12% as uncoordinated structures [34]. Ly is defined as a 1,4-β-N-acetylmuramidase. Ly cleaves the glycosidic bond between N-acetylMuramic acid and N-acetylglicosamine in bacterial peptidoglycans, which constitute the major part of the bacterial cell wall. There are two types of lysozymes [35]: those found in hen egg-whites, also known lysozyme c and those found in Embden goose egg-whites or lysozyme g. However, it is unclear whether bovine Ly is a type c or g Ly. Both types c and g Ly are present in bovine stomach tissues and fluids [36], raising the possibility that both types of Ly are present in bovine milk. In addition, there are similarities between milk Ly and La in 3-dimensional structure [37].

1.2.1.7. Lactoferrin

With respect to physiological function, lactoferrin may be the most important protein in milks, despite its very low content. Lf is one of the members in the transferrin family which have attracted extensive studies on their structures and functions. The primary members of the transferrin (Tf) family, including serum transferrin (sTf), ovotransferrin (oTf, from egg white), and lactoferrin (Lf, from milk, other secretory fluids, and white blood cells), are monomeric glycoproteins, MW = 80 kDa, (670-690 residues), with the capacity to bind very tightly, but reversibly, two Fe³⁺ ions together with two CO₃²⁻ anions [38]. Two of the most widely studied members of this group are Lf and sTf, and the amino acid sequences of theirs are well established [39, 40, 41]. Proteins of the transferrin family have high sequence homology (identical residues in corresponding positions), which shows
59% between human lactoferrin (hLf) and human serum transferrin (hsTf). The lactoferrins from different sources show even higher homology. The sequence of HLf possesses 69% homology with that of bLf (bovine lactoferrin) [40, 42] and 70% homology with that of mice lactoferrin [43]. The sequence of bLf from species Bos Primigenius Taurus is given in Fig.1-3.

The three-dimensional structures of the proteins in the transferrin family have been successfully determined by X-ray crystallography. X-ray crystal structures have been reported [38] for human lactoferrin (hLf) in apo, diferric, dicupric, and oxalate-substituted forms, and rabbit serum transferrin (rsTf) as the diferric molecule and the ferric N-lobe half-molecule.

The structure of human lactoferrin has been determined in greater detail as shown in Fig.1-4, and details are described as follows [38, 44, 45, 46]. The protein is folded into two equal size lobes with very similar structure. The two lobes comprise residues 1-332 (N-lobe) and 344-703 (C-lobe). N- and C-lobes are divided into 4 domains (N1, N2, C1 and C2). The iron binding sites in the N- and C-lobes are remarkably similar and are exquisitely designed for Fe$^{3+}$ and CO$_3^{2-}$.

Three anionic ligands, which are the same in both sites are Asp61, Tyr93 and Tyr191 (Asp407, Tyr447 and Tyr540 in the C-lobe), match the 3+ charge on the metal ion; the fourth ligand is a neutral His252 (His609 in the C-lobe). The charge on the CO$_3^{2-}$ is almost exactly matched by positive charge at its protein binding site, the Arg site chain (+1) and N-terminus of a helix (number 5), with a charge of at least +0.5. The anion fits perfectly into a pocket between the metal and the protein and forms an elegant hydrogen-bonding network involving the Arg residue, a
conserved Thr on the loop preceding helix 5, and main chain N-H groups from the N-terminus of helix 5.

The carbohydrate chains on transferrins appear to be all N-linked through Asn residues. Glycosylation sites vary in number (from one in rsTf and chicken oTf to four in bLf) and are scattered over the molecular surface, arguing against any direct structural or functional role. The carbohydrate sites of lactoferrin identified so far include residues 137, 416, 478, 623 for hLf and residues 233, 368, 478, 547 for bLf (see Fig. 1-3 and 1-4). All of the lactoferrin contains biantennary glycans of the N-acetyllactosamine type α-1,6-fucosylated on the N-acetylgalactosamine residue linked to the peptide chain. Only hLf contains α-1,3-fucosylated N-acetyllactosamine residues [47]. bLf is characterized by glycans possessing α-1,3-linked galactose residues in the terminal non-reducing position and by N-acetylgalactosamine residues replacing galactose residues [47].

The tertiary fold of transferrins (the proteins of transferrin family) into two homologous lobes, each with two dissimilar domains, gives ample scope for molecular flexibility, and this appears to be a vital factor in their function [38]. Small-angle X-ray and neutron solution scattering [48] and other physical measurements [49] have indicated that a large conformational change accompanies iron uptake and release by transferrins. The nature and extent of this change has been graphically demonstrated by the X-ray structure analysis of apo-human lactoferrin [45], where a large-scale domain movement in which domain II in the N-lobe rotates about 540° relative to domain I about a hinge at the back of the iron-binding site, causes the binding cleft to open wide.
This structure suggests a plausible model for the mechanism of iron-binding, in which the metal first binds to domain II (N2 or C2), with the cleft open, after which the domain closes over the metal to complete its coordination [44]. A curious feature of the apo-hLF crystal structure is that whilst the N-lobe is open, the C-lobe is closed, even though it has a metal bound. A likely explanation is that, given the flexibility of the molecule in solution, an equilibrium exists between the open and closed forms of the apoprotein, with binding only occurring with the open form (Fig.1-5).

Although structural study by X-ray diffraction of bLF is in progress [38], the analyses by partial proteolytic hydrolysis have demonstrated that same characteristics of the tertiary structures exist in hLF and bLF such as bilobal molecule [50, 51, 52], and iron-binding properties [53, 54]. In addition, the study by superposition of the N1-domain peptides of bLF and hLF (covering the amino-acids residues 4 to 52), have indicated that the conformation of the two peptides are identical [55]. Linked with the binding or killing properties of this N1-domain peptide (of bLF and hLF) to animal and microbial cells, hLF and bLF should play similar biological roles in vivo [55].

1.2.1.8. Other Enzymes

Whey was reported to have some oxidoreductase enzymes, notably xanthine oxidase, sulphydryl oxidase and superoxide dismutase [56, 57, 58]. Table 1-5 shows some information on these enzymes. However, the protein structures of the enzymes are unclear.

1.2.2 Structure of Lactose
Lactose is a disaccharide that contains D-glucose and D-galactose linked by a β-1,4-glycosidic bond. It is designated as 4-O-β-D-galactopyranosyl-D-glucopyranose.

1.2.2.1 Mutarotation

Lactose normally occurs naturally in two forms, α-monohydrate and anhydrous-β, which differ in their steric configuration of the H and OH around the C-1 of the glucose. When either form of lactose is dissolved in water, there is a gradual conversion of one to the other until equilibrium is established [65].

\[ \begin{align*}
\alpha\text{-D-lactose} & \xrightarrow{k_1} \beta\text{-D-lactose} \\
\beta\text{-D-lactose} & \xrightarrow{k_2} \alpha\text{-D-lactose}
\end{align*} \]  

The equilibrium ratio of $\beta/\alpha$ at 20°C is $62.7 / 37.3 = 1.68$.

Mutarotation has been shown to be a first order reaction characterised by the reaction constants $k_1$ and $k_2$. If a dilute lactose solution at constant temperature contains $a$ moles of $\alpha$- and $b$ moles of $\beta$-, then the amount of $\beta$-formed ($x$) per unit of time is

\[ \frac{dx}{dt} = k_1(a-x) - k_2(b+x) \quad (1-2) \]

The mutarotation coefficient ($k_1 + k_2$) can be determined by the change in optical rotation with time:

\[ k_1 + k_2 = \frac{1}{t} \log \frac{r_{0} - r_{\infty}}{r_{t} - r_{\infty}} \quad (1-3) \]

where $r_0$ is the optical rotation at zero time, $r_t$ is the rotation at time $t$ and $r_{\infty}$ is the equilibrium (final) rotation. Plotting the difference in rotation at time $t$ and at
equilibrium \((r_t - r_w)\) against time gives a straight line with a slope equivalent to the mutarotation coefficient [65, 66].

1.3 Isolation of Whey Proteins and Lactose

1.3.1 Isolation of Whey Proteins

Over the past 20 years, nearly all available techniques for protein separation have been used to isolate and fractionate whey proteins. Traditionally, the product known as Lactalbumin, which results from heat-induced precipitation of whey protein, was produced commercially in several countries. Total manufacture was small, however, because denaturation of the proteins resulted in limited applications for the product in formulations of food products. The lactose industry also produced an early type of whey protein concentrate in the form of a mother liquor, which has been used for many years as a stock food [61]. I refer to these products as the First Generation of Whey Protein.

The commercial success of the Second Generation of Whey Protein, known as Whey Protein Concentrate (WPC) and Whey Protein Isolate (WPI), obtained by the use of ultrafiltration and ion exchange, has resulted in increasing production in recent years. In 1990, the United States food industry manufactured approximately 173 million pounds of whey protein concentrate that contained 35-75% protein and a smaller but unknown amount of whey protein isolate that contained approximately 90% protein [62].
Current industrial procedures for manufacturing WPC usually involve the following series of processing treatments: (1) fresh cheese whey is pretreated to remove insoluble materials including fat, casein residues and colloids; (2) the pretreated whey is concentrated by ultrafiltration (UF) to achieve a volume concentration ratio of 20-25%; (3) the UF retentate is concentrated by vacuum evaporation (optional); and (4) the UF retentate (concentrate) is spray dried [68]. The more the retentate is concentrated by UF, the higher the protein content in the product. Using only UF, it is difficult to concentrate the protein, although concentration to 80% protein can be achieved by using diafiltration [63].

A WPI has been produced since 1985 by an ion exchange process, developed by Bioisolates Ltd., UK (BIL), in the plant of the Le Sueur Cheese Co., MN, USA (LSI) [63, 64] which is processing 1,250,000 lb of whey per day. In the BIL system, the whey is mixed with regenerated cellulose (sulphopropyl) ion-exchanger in a stirred-tank at pH 3.2. The ion-exchanger adsorbs the protein and the deproteinized whey is then removed; the ion-exchanger is washed with water, then the protein is desorbed into water by changing the pH to 9. The desorbate contains almost pure protein. This pure protein solution is concentrated by UF and spray-dried. Three commercial WPIs and eight commercial WPCs, which were made in Denmark, US, UK, West Germany and New Zealand, were recently examined for a comparison of composition, functionality and flavour by Morr et al, [65]. As a result, WPIs have shown an improvement in all aspects, functionality and in quality, and overcome the disadvantages with WPC.
The ultimate performance of a whey protein complex such as WPC and WPI will be influenced by either the synergistic or antagonistic properties of individual protein components. Some whey protein in the complex, e.g. enzymes, Lf and Igs which are often referred to as "physiologically functional proteins", if isolated, can play a more important role in other fields such as functional foods. Studies on fractionation of whey proteins [66, 67] have indicated that the functional properties of individual fractions of whey proteins differed in important ways.

Thus, the Third Generation of Whey Protein, i.e. protein ingredients based on effective fractionation of whey, will be the commercial products of the near future. Many important techniques available for the separation of protein have been tried in the fractionation of whey. Some of the methods have already been developed to a commercial scale. Pearce [68] developed a process of thermal precipitation to selectively enrich Lg in the supernatant. In order to remove Lg, a major allergenic component of bovine milk, many methods have been introduced. The addition of 4 mM FeCl₃ to cottage cheese whey at pH 3.0 precipitated all the protein except Lg [69]. When acid whey was treated with FeCl₃ (7.5 mM, pH 4.3, 4°C), 90% of the Lg was precipitated with BSA while 70% of the Igs and 95% of La remaining in supernatant [70]. When Cheddar cheese whey was treated under optimized conditions, i.e. 1.33 mg sodium hexametaphosphate/ml at pH 4.07 and 22°C for 1 hr., greater than 80% of Lg was removed by precipitation. In the supernatant, almost all of the Ig and La were retained [71]. Selective adsorption of β-Lg on CM-cellulose resin could be performed at pH 4.6 in the case of whey dialysed for 48 hr. Lg accounts for less than 20% of major proteins in the Lg-eliminated fraction, and
greater than 90% of Lg of major proteins in the Lg rich fraction [72]. Lg has also been selectively removed from whey by high pressure-induced proteolysis using thermolysin [73, 74].

Whey can also be fractionated using ultrafiltration membranes with different molecular weight cut-offs. Therefore, high-molecular weight proteins such as Lf, Lp and Ig can be separated from low-molecular weight whey proteins by an one or two stage ultrafiltration [75, 76].

Chromatography in general is a more powerful method, yielding finer fractionations of the proteins than membrane filtration. Those biologically important proteins such as Lf, Lp and Igs were mainly isolated by chromatography. Lf and Lp, or Lf and Ig have been isolated from whey by CM-containing cation exchangers [77, 78], SP-containing cation exchanger [79], copper chelating chromatography [80], gel filtration chromatography [81], cellulose phosphate resin [82], and microfiltration affinity purification [83]. Higher purities and yields for Lf have been obtained as a purity of 96% and the yield of average 80% was obtained by Foley and Bates [82]. A 90% recovery and 95% purity were reported by Dionysius et al. [78] and Chen and Wang [83], respectively. Microfiltration affinity seems to be a more powerful technique for isolating IgG with 90% purity and 86% activity [83]. In contrast, the purity of IgG in the Ig rich fraction obtained by chelation chromatography was 77.2 and 53.0% for acid whey and Cheddar cheese whey, respectively [80].

Fractionation of the macropeptides has traditionally been achieved by precipitation with increasing concentrations of trichloroacetic acid (TCA) since glycomacropeptide (GMP) is preferentially soluble in 12% TCA solution [84]. More
and Seo [26] have demonstrated procedures for isolating GMP from the TCA-soluble fraction of chymosin-hydrolyzed casein by gel filtration, and ion exchange chromatography. Tanimoto et al. [85] have developed a procedure which could isolate GMP on an industrial scale based on membrane filtration and Q-sepharose ion-exchanger chromatography with a yield of 8.1 mg powder / kg whey and a purity of 88%. Another method for GMP separation on a commercial-scale was developed by Kawasaki et al. [86] based on ultrafiltration and diafiltration.

1.3.2 Isolation of Lactose

Crystallization is a widely used method to isolate lactose from whey or permeate. In general, about 50% of the lactose is recovered from whey [87].

More recently, permeate has become a useful raw material for lactose [88]. The process for lactose crystallization is based on three major steps: concentration, crystallization, and separation. Whey can only be concentrated by evaporation to 58-62% total solids. Temperature must be less than 70°C to avoid protein denaturation and viscosity problems. Permeate, on the other hand can be preheated up to 90°C to reduce precipitation and also can be concentrated to 65% total solids. As the crystallization process is affected by concentration, crystallization time is shorter and the yield greater from the permeate [87]. Purification of lactose from the permeate is also simpler; a good quality product with 99.6% lactose monohydrate can be obtained [4].

Ion exclusion chromatography was developed by Wheaton and Bauman [89] in 1953. This discovery is based on the observation that certain ion exchangers, in particular, cation exchangers, when their functional group is saturated with counter-
ion, allow dissolved, but non-ionized solutes such as sugar to penetrate freely into the interior of the resin particle, while rejecting electrolytes to the exterior of the resin. This method has found applications for both laboratory and industrial scale separations.

Ion exclusion has been used for the commercial purification of molasses [90, 91]. A sugar recovery of about 77% in the product and a non-sugar rejection to the waste of about 80% was reported from those studies. Overall dilution was about 814% on original 80 Bx feed molasses. In actual commercial operation, completing 99 cycles over a five day operation period, recovery of crystalline sugar was reported at 58.3% on the sugar in the feedstock. Losses to secondary molasses were 19% and about 22% to the non-sugar fraction [92].

The ion exclusion technique is also utilised in the sweetener industry for separation of D-glucose and D-fructose in high fructose syrups. Even though the mechanism of the chromatography process is complicated, the operation is relatively simple, where a small volume of a syrup containing a mixture of both fructose and glucose is placed on top of a bed of the calcium form of a suitable cation exchange resin and slowly displaced with water. The glucose will move more rapidly down the column while the fructose is retarded since the resin has a higher affinity for the latter [93]. The purity of the rich fractions of glucose and fructose is between 95 and 97% calculated from a dry material basis [94]. In spite of the great practical interest in the separation of glucose and fructose, there are only a few reports covering their separation in industrial processes [95].
More recently, separations of lactose from milk [96, 97, 98] and whey [99] by ion exclusion were reported. In the processes for chromatographically fractionation of milk, whey and mother liquor at a temperature of about 50 to 75°C and a pH of about 5.5 to 7, sulphonated polystyrene resin which is in sodium ion form (Na-form) and 3 - 6% by weight divinylbenzene crosslinker is used. The average grain size used was about 0.2 to 1 mm; the flow rate was about 0.4 to 1.5 m³/h, and elution was carried out with water. The following fractions were recovered, a) a protein fraction, b) an intermediate fraction comprising lactose and other impurities for recirculation. c) a lactose fraction. 75% of the lactose was recovered from the lactose fraction. The total yield was 92.5% when the recirculation of the intermediate fraction was included.

1.4 Functionality of Whey Proteins and Lactose

Even though the terminology or definition of "functionality" is highly inconsistent and confusing, whey components are categorized by three functionality terms in this thesis, namely physicochemical, nutritional, and physiological functionality.

1.4.1 Physicochemical Functionality (Functional Properties)

1.4.1.1 Whey proteins

The term "functional properties" is commonly used to describe 'those physical and chemical properties which affect the behaviour of proteins in food systems during processing, storage, preparation and consumption' [100]. Some excellent
reviews have been written on the functional properties of whey protein [10, 101, 102]. Typical functional properties of proteins in model food systems are shown in Table 1-6.

1.4.1.2 Lactose

Physicochemical functionality of lactose is concerned with properties such as sweetness; flowability and anti-caking qualities; affinity for surface adsorption of colors and flavor volatiles; wettability, dispersibility and solubility; tableting properties [104]. The solubility, moisture sorption and sweetness vary with its mutarotation and crystalline forms. β-Lactose has higher initial solubility and sweetness than α-lactose. The tightly packed molecules in a pure crystalline system adsorb very little moisture while amorphous glass lactose adsorbs much more water than the crystalline forms [105].

1.4.2 Nutritional Functionality

Nutritional functionality in a broad sense embraces some parts of physiological functionality. However, the definition of nutrition is used here as " the process by which food and other substances eaten become part of the organism; the food humans eat enables them to live, grow, keep healthy and well, and get energy for work and play". In other words, nutritional functionality here deals with those nutrients for enabling humans to live rather than "regulating bodily processes".

1.4.2.1 Whey Proteins

The whey proteins are some of the most nutritious proteins available, and their excellent nutritional value could be utilized to a greater extent [101].
The high nutritional value of whey protein is based on its high concentration of essential amino acids compared with most other proteins (Table 1-7). Whey protein has all the essential amino acids in excess of FAO standards: of particular importance are isoleucine, lysine, threonine and tryptophan [106]. Whey protein has an even higher concentration of tryptophan, leucine, threonine and lysine than whole egg protein. Table 1-8 lists an evaluation of the nutritional value of different food proteins. So, whey protein should also be promoted in applications based on their nutritional profiles. Unfortunately, their primary function to date has been to impart specific physicochemical properties to the finished product [107].

1.4.2.2 Lactose

Lactose is the principal carbohydrate of all mammalian milk. except for a few pinnipeds like sea lions [109]. Nutritional value of lactose seems negative because approximately 70% of the world’s population has difficulty digesting lactose [109].

Digestion and absorption of lactose in humans involves hydrolysis into glucose and galactose by lactase (β-galactosidase), a membrane-bound enzyme present in the brush border of the small intestine’s epithelial cells. If the amount of lactose ingested exceeds the hydrolytic capacity of the available intestinal lactase, a portion of the lactose remains undigested and is transported into the large intestine. Undigested lactose in the large intestine increases the osmolality of the intestinal fluid and thus draws water from the tissues into the intestine. Undigested lactose may also be fermented by bacteria in the colon, thus generating organic acids, carbon dioxide and hydrogen. These fermentation products together with the large amount of water drawn into the intestine are largely responsible for various symptoms [110].
1.4.3 Physiological Functionality (Biological Functionality)

Milks are secreted by mammals (> 4000 species) for the primary function of providing the complete nutritional requirements of the neonate of the species. However, by accident (e.g. mechanism of excretion), but more probably by design, milk serves other functions in addition to being strictly nutritional [111]. Most of the components that have non-nutritional functions, or physiological functions, remain in the whey. Components of whey that have been recognized as contributing to physiological function are included in Table 1-9.

1.4.3.1 Total Whey Protein

Total whey protein containing La, Lg, Lf, Lp, BSA and lgs, have biological effects when ingested, including an anti-cancer action [113, 114]. Results have established the superior action of dietary whey protein relative to other common protein sources (casein, meat, fish, soybean) in retardation of colon cancer using a rat model. Whey protein-fed animals displayed the least tumor burden and tumor number of the five dietary groups, with only 20% of animals developing tumors in the colon. Splenic glutathione levels were at least 25% higher for the whey-fed rats than those receiving the other diets [114].

Total whey protein also exhibits a greater hypocholesterolemic effect in comparison with casein or soybean protein, while soybean protein had no hypocholesterolemic action compared with casein. The cholesterol-lowering action of whey protein was mainly attributable to the decrease in LDL + VLDL-cholesterol. The liver total lipids and cholesterol levels in rats fed with whey protein were
significantly decreased in comparison with those in rats fed with casein and soybean protein [115].

1.4.3.2 α-Lactalbumin and β-Lactoglobulin

It is well known that La is a subunit of lactose synthetase, the modifier protein for galactosyltransferase in lactose biosynthesis. In addition, the characteristics of the La Ca\(^{2+}\)-binding site imply that its function in vivo is regulatory. Recently acquired evidence also indicates that Ca\(^{2+}\) plays an important regulatory role in the endoplasmic reticulum [15]. A biological function for Lg has been sought for some time. The protein is capable of binding several hydrophobic molecules, including retinol, and it has been proposed that \textit{in vivo} Lg functions to protect retinol against oxidation and to transport it through the stomach to the small intestine [14, 111].

1.4.3.3 Immunoglobulins

Bovine Ig has been found to be an ideal antagonist to those diseases caused by microbial infection. Colostrum or milk with high concentrations of Ig from immunized cows has been proved to be effective in the treatment of \textit{E. coli} [116, 117], \textit{Cryptosporidium} [118], and rotavirus with minimal side effects [119,120]. Infectious gastroenteritis by \textit{E. coli} and rotavirus have interested public health scientists for many years. Enterotoxigenic \textit{E. coli} [117] is the organism most frequently associated with diarrhoea in travellers to less-developed countries, accounting for 30 to 70\% of cases. Rotavirus infection is also the major cause of infectious diarrhoea in infants and young children, and accounts for about half of the paediatric hospital admissions of children with acute diarrhoea in western Europe and
the United States [119]. In Australia, about 95,000 children under three years are rotavirus-infected every year [121]. One of the most important problems with rotavirus infection is their prevention. Antibiotics are ineffective for a virus, and vaccine development is at least another ten years away [121]. So, passive immunization with bovine IgG is an alternative approach to resist rotavirus infection.

On the other hand, the regulation of the immune system by cow milk IgG has been studied. Kulczycki et al. [122] reported that cow milk IgG inhibited markedly pokeweed mitogen-induced antibody secretion by human peripheral blood mononuclear cells. Miura et al. [131] observed that cow milk IgG1 had mitogenic activity on mouse splenocytes.

1.4.3.4 Lactoperoxidase and Other Enzymes

Lp itself has no antibacterial effect but in combination with certain co-factors forms a potent antimicrobial system. These co-factors include thiocyanate and hydrogen peroxide. Lp catalyses the oxidation of thiocyanate by hydrogen peroxide to form oxyacids of thiocyanate; i.e., OSCN⁻ and O₂SCN⁻. The antibacterial mechanism is caused by oxidation of vital SH groups by OSCN⁻ and O₂SCN⁻ in vital metabolic enzymes, e.g., hexokinase, glyceraldehyde-3-phosphate-dehydrogenase, and depletion of reduced nicotinamide adenine nucleotides [29]. Another mechanism [124] seems to be based on the observation that OSCN⁻ appears to have a chaotropic effect on the inner membrane of bacteria, resulting in leakage of potassium ion and amino acids, plus the inhibition of uptake of carbohydrates, amino acids, etc. thereby stopping the synthesis of protein, DNA and RNA.
A wide variety of bacteria can be inhibited or killed by Lp-system, which include many Gram-positive bacteria such as lactococci and lactobacilli [125, 126] and Gram-negative bacteria such as *E. coli*, *Pseudomonas* spp. and *Salmonella* spp. [127, 128].

The oxidoreductases in whey such as xanthine oxidase, sulphydryl oxidase and superoxide dismutase, are all concerned with the production of hydrogen peroxide. Xanthine oxidase is often thought of as the donor of hydrogen peroxide to the Lp system in milk. Many characteristics of these enzymes are unknown, similarly their potential functions of biology.

The antibacterial characteristics of bovine Ly are similar to those from other species. Gram-positive bacteria are thus generally more susceptible because their cell-walls are composed to a major extent (90%) of peptidoglycan. With Gram-negative bacteria the peptidoglycan content is much lower (5-10%) and they are protected by an outer layer of lipopolysaccharide, which prevents lysozyme from reaching its substrate.

**1.4.3.5 Lactoferrin**

Probably no other milk protein has shown such wide biological functions as lactoferrin. The diversity of functions proposed for Lf (Table 1-10) makes it very difficult to draw any overall conclusion about the role of this protein [129]. The controversial nature of some reports, and the essentially "phenomenological" nature of others further clouds the issue [129]. Diversified binding properties of lactoferrin may, however, be critical to its function, namely its ability to bind iron extremely tightly even at low pH, and its readiness to bind to a wide variety of cells and other
molecules [129]. Reported biological functions of lactoferrin (mainly hLf and bLf), are listed in Table 1-10 and details are discussed as follows.

1.4.3.5a) Antimicrobial Activity

The antibacterial action of Lf was first reported by Bullen [130]. In vitro study suggested that human apolactoferrin bacteriostatically inhibits the growth of E. coli by depriving the cells of iron essential for their growth. On the other hand, Arnold et al. [131] have shown that Lf is capable of a direct bactericidal effect on Streptococcus mutans, Vibrio cholerae and certain other strains. Ellison et al. [132] have reported that Lf and transferrin cause the release of lipopolysaccharide from the gram-negative outer membrane and so change the membrane permeability. A direct interaction between Ly and Lf was observed with Micococcus luteus by Perraudin and Prieels [133]. They found that protoplasts produced by the lytic activity of Ly were strongly agglutinated by Lf. Suzuki et al [134] demonstrated that Lf and Ly inhibit the growth of E. coli 0111 regardless of the degree of iron saturation of Lf. The agglutination was clearly observed with cells prepared from a medium containing Ly and iron-saturated Lf, but not from a medium containing Ly alone. Ellison et al [135] have found that while each protein (Lf or Ly) alone is bacteriostatic, together they can be bactericidal for strains of V. cholerae, S. typhimurium, and E. coli.

An anti-virus effect of Lf was also investigated in which bLf inhibited the influenza virus-induced hemagglutination of red blood cells that may protect us from the virus infection [136].
When lactoferrin, either bovine or human, is treated by an acid protease such as pepsin, the antibacterial activity of the resultant hydrolysates increases considerably compared with the original intact Lf [137, 138]. The active peptide [138], "lactoferricin", was found to consist mainly of a loop of 18 amino acid residues formed by a disulfide bond between cysteine residues 20 and 37 of human Lf (lactoferricin H), or 19 and 36 of bovine Lf (lactoferricin B). Lactoferricin is highly effective against a broad range of Gram-positive and Gram-negative bacteria including pathogenic strains of *Listeria*, *E.coli*, *Pseudomonas aeruginosa*, *Salmonella*, and *Campylobacter*, but not against many strains of beneficial bacteria, such as *Bifidobacterium* [139]. In addition, lactoferricin B has also shown a fungicidal effect of killing the yeast *Candida albicans*. Its effect was lethal, and caused a rapid loss of colony-forming capability [140].

The bactericidal or fungicidal effectiveness of either lactoferrin or lactoferricin is inhibited in the presence of Mg$^{2+}$ or Ca$^{2+}$ ions [132, 135, 140, 141]. Although the mechanism of antimicrobial activity is not fully defined, observations have suggested that killings of bacteria and yeast by lactoferrin and lactoferricin are based on their binding with the microbial cells (see section 1.4.3.5h).

1.4.3.5b) Growth factor of animal cells

Lf has been found as the stimulating factor of animal cells [139]. Nichols et al. [142] found that human colostrum stimulated a significant increase in thymidine incorporation into rat crypt cell DNA and identified the stimulating factor as Lf. It has been shown that Lf promotes the proliferation of certain cell lines. The DNA synthesis-stimulating activity of holo-Lf from both human and bovine milk has been
demonstrated also using BALB/c mouse 3T3-3K embryo fibroblast cells [143]. It has been shown that Lf is an essential growth factor for human lymphocytic cell lines [144]. The uptake and resecretion of human Lf by human B-lymphocytes have been demonstrated by electron microscopy using anti-human Lf and gold probe, and by means of a reverse hemolytic plaque assay using anti-Lf serum, respectively [145]. Stimulation by bovine Lf of nerve growth factor (NGF) synthesis/secretion in mouse L-M cells has been studied, where both apo- and holo-bLf induced an increase in NGF content in the cell-conditional medium with similar effectiveness. Neither apo- nor holo-bTf was effective [146].

1.4.3.5c) Effect on the immune system

There have been many reports [129] indicating that Lf can affect various aspects of immune function. The effects reported include both inhibition [147] and stimulation [148] of antibody synthesis, enhancement of monocyte cytotoxic activity [149, 150], inhibition of the activity of the C₃ component of complement [151], enhancement of helper T cell development in the thymus [148], modulation of lymphocyte proliferation [152, 153, 154, 155] and regulation of granulopoiesis [156, 157].

1.4.3.5d) Inflammation

It was proposed several years ago that Lf might contribute to the hypoferremia of inflammation [158]. It was suggested that Lf released by degranulating neutrophils could remove iron from circulating Tf, and transport it directly to macrophages of the reticuloendothelial system, where it would be incorporated into ferritin [129].
1.4.3.5e) Anti-oxidation

Iron catalyzes the generation of hydroxyl radical (·OH) from hydrogen peroxide and superoxide radicals (O$_2^-$). When the highly reactive ·OH reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxide (LOOH) is produced [159]. Lipid peroxidation also occurs in the presence of ascorbic acid and iron ion even in an absence of hydrogen peroxide [160]. The carbonyl products converted from LOOH are responsible for DNA damage, generation of cancer, and aging-related disease [161].

The suppression of lipid peroxidation by Lf has been reported by Winterbourn et al [162] with PMA-stimulated neutrophils. Baldwin et al [163] suggested the possibility that Lf promoted the formation of hydroxyl radicals when the concentration of Fe$^{2+}$ exceeded a certain level where Lf chelates iron completely. A recent paper has indicated that bovine apo-Lf is able to inhibit the accumulation of TBARS in iron/ascorbate-catalyzed lipid peroxidation not only in liposomes, but also cultured cells and mouse tissue homogenates [164].

Additional anti-oxidant and immunomodulatory effects of bLf in human have been investigated [165]. bLf inhibits the free radical generation by normal human monocytes in response to PMA. This effect on free radical generation is linked to the nature of the divalent cationic metal that combines with the apo-Lf. In addition to its anti-oxidant effect, Lf stimulates, in combination with lipopolysaccharide (LPS), the production of cytokines and of prostacyclin by normal human monocytes. This potentiating effect is independent of the cation that substitutes the Lf, since the apo-Lf is able to induce it [165].
1.4.3.5f) Anti-cancer

An investigation of the antitumor effects of Lf [166] found that Lf inhibits growth in mice of transplantable solid tumors induced by v-ras transformed fibroblasts and a methylcholanthrene-induced fibrosarcoma. Lf also substantially reduced lung colonization (metastasis) by B16-F10 melanoma cells in syngeneic mice. Iron-saturated and apo-Lf exhibited comparable levels of tumor inhibition and antimetastatic activity. Tf had no effect on lung colonization. In addition, Lf has been shown to regulate the release of tumor necrosis factor alpha and interleukin 6 in vivo [167].

1.4.3.5g) Inhibition of Cholesterol Accumulation

When macrophages are incubated with acetylated or oxidized low density lipoproteins (Ac- or OxLDL), cellular cholesteryl esters (CE) increase significantly. Kajikawa et al. [168] investigated the effect of whey protein on Ac- or OxLDL mediated accumulation of CE in macrophages and found that Lf inhibits the accumulation of CE dose-dependently. In the presence of bLf (1mg/ml), CE accumulation in macrophages incubated with AcLDL (100mg of protein/ml) decreased by more than 80%. hLf was less potent than bLf, and bTf had no effect.

1.4.3.5h) Diversified Binding Properties

Despite being widely regarded as an iron-binding protein, lactoferrin can actually bind many different molecules, a characteristic critical to its function [129]. To date Lf, mainly involving hLf and bLf, has been reported to bind with metal ions, proteins, cells and DNA, as discussed below.
A) Metal-binding

Besides iron Lf is known to bind Cu²⁺ [38], which is also a transition metal. A larger cation, hafnium, has been also studied for binding with hLf and bLf. Two specific binding configuration were observed [169]. The studies of human serum Tf and hen’s egg conalbumin by ultraviolet difference spectra [170, 171] indicated that many transition metals, as well as Ga²⁺, Al³⁺ and VO²⁺ can bind to these iron-binding proteins. hLf was reported [172] to form self-associate tetramers in the presence of calcium.

B) Protein-binding

Lf was reported to interact with many milk proteins and enzymes such as casein and albumin [173], secretory IgA [174], lysozyme [175] and β-lactoglobulin [176].

C) Cell-binding (Lactoferrin Receptor)

——Animal Cell

Binding of Lf to different blood cells has been previously analyzed by several authors [177, 178, 179, 180]. bLf and hLf were also reported to bind with other animal cells such as human platelets [181], human promonocytic cell line U937 [182], and the cional MAC-T bovine mammary epithelial cell line [183].

Human lactoferrin receptors have been identified on different target cells such as enterocytes, activated human lymphocytes, platelets and breast epithelial cancerous cell lines. All these receptors consist of a single polypeptide chain of about 110 kDa [55]. The receptor binding site of Lfs from different origins is located in the N-lobe of the molecule covering the amino-acids residues 4 to 52 [55].
The conformation of this 4-52 N-lobe peptide of hLf and bLf is very similar indicating that bLf is able to recognize hLf receptor and to compete with hLf molecules [55]. Glycans of Lfs from different origins are not involved in the binding to human lactoferrin receptors.

Lf receptor in piglet small intestine has also been detected as specific and saturable binding of $^{59}$Fe-labeled pig lactoferrin by brush border membranes purified from piglet intestine [184].

———Microbial Cells

The organisms such as *Neisseria spp* [185, 186] and *Haemophilus influenzae* [187] express outer membrane proteins which act as receptors for Lf itself. An iron limitation-inducible outer membrane protein of *Neisseria meningitidis*, the iroA gene product, has been identified as a lactoferrin receptor [188]. As a first step in localizing the regions of human Lf involved in binding to bacterial lactoferrin receptors [189], N-lobe and C-lobe fragments were assessed for binding to receptors on *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Moraxella (Branhamella) catarrhalis*. Solid phase binding studies demonstrated that the isolated C- and N-lobe preparation were capable of binding to membranes from iron-deficient *N. meningitidis*, *N. gonorrhoeae* and *M. catarrhalis*. The binding of the individual C- and N-lobes was confirmed by an analytical SDS-PAGE binding method in which the membrane-associated polypeptides were identified by prior biotinylation and subsequent binding of labelled streptavidin [189]. By studying interaction of lactoferricin B (amino acid residues 17-41 from N lobe of bLf) with the microbial cells, $^{14}$C-labeled peptide has been found to be bound rapidly to the surface of *E.coli*
and *B. subtilis* [190] and to *C. albicans* [140]. The rates of binding were consistent with the rates of killing induced by the peptide.

(D) DNA-binding

Recent research shows that Lf enters the cell from the serum and is transported into the nucleus where it binds DNA [191]. The interaction of Lf with the DNA sequences was studied [192] by several methods to reveal the characteristics for lactoferrin binding to DNA [191]. First, the binding was strong (*K_d = 1.24 x 10^{-8}* ) and cooperative; saturating molar ratios of Lf bound to DNA in complexes of 2:1 and 4:1 at low and high Lf concentrations, respectively. Second, iron-saturated Lf bound DNA more avidly than did apo-Lf. Finally, there was a broad but specific interaction between Lf and the DNA consensus sequences. This is consistent with the notion that more than one lactoferrin molecule binds to the DNA sequences. A new role of Lf has been further found [192] that the extracellular protein lactoferrin may regulate gene transcription. This potential direct transcriptional function of lactoferrin is a unique mechanism for gene activation, which findings identifies a new class of transcriptional activators that are secreted by one cell, are taken up by a target cell, and are then transported intact to the nucleus where they activate gene expression [191]. These observations can be used for explaining the molecular basis for Lf's role in the inflammatory response and in the transfer of immunity from mother to child [191]. First, during the response to infection, after Lf has been released from granulocytes, has bound iron, and has been internalized by other cells, Lf may act to modulate the immune response. Second, Lf in milk could be directly involved in the stimulation of the newborn's immune system.
1.4.3.6 Glycomacropeptide and Proteose-peptone

Some biological functions of GMP have been reported, i.e. inhibition of gastric secretion [193], periodic contractions of the stomach and duodenum [194], aggregation of ADP-treated platelets [195], adhesion of oral *Actinomyces* and *Streptococci* to erythrocytes or polystyrene [196], inhibition of the binding of cholera toxin to its receptor [197] and suppression of influenza virus haemagglutination [198].

Acid whey inhibits up to 90% of iron-catalyzed oxidation of phosphatidylcholine liposomes. Ultrafiltration, dialysis, heat treatment and chloroform extraction indicated that the primary antioxidant(s) in acid whey were polar, heat-stable compound(s) with molecular weights between 500 and 5000. A potential antioxidant peptide in whey in this molecular range is proteose peptone 8-fast [199].

1.4.3.7 Lactose

The physiological effects of lactose in the diet have become of major interest to health professionals because of lactose intolerance. Hourigan [110] did an extensive review on the physiological effects of lactose.

The question arises of why it is lactose rather than other easier-metabolized carbohydrates that is evolutionally predominant in the milk of most mammals if it has only the common nutritional role of providing energy. A reasonable answer if that lactose has many biological functions besides nutrition.

Lactose appears to stimulate the intestinal absorption and retention of calcium [110]. The effect may not be due to lactose but rather to its metabolic product, lactic
acid, formed by microbial action in the gut [60]. In addition to calcium, lactose also enhances the absorption of magnesium, phosphorus, and other essential trace elements [104]. Recently it was reported that lactose dramatically increased iron absorption in rats [200].

Lactose in the diet is useful in the desired balance of intestinal flora. By promoting a more desirable flora in the lower digestive tract, it is effective in combating gastrointestinal disturbances caused by putrefactive bacteria, as well as in promoting synthesis of the B vitamins for absorption by the host [104, 199, 201]. Lactose is useful in chickens for the promotion of growth and body weight, and reduction of body fats in chickens [202].

It has also reported [203] that interaction of two biologically important proteins isolated from HL-60 cell nuclear extracts, CBP35 and CBP70, can be altered by a conformational change in CBP35 induced by the binding of lactose to its carbohydrate-recognition domain. On the other hand, glucose failed to have the same effect as lactose. This might indicate that lactose has some unknown regulating functions.

Due to its slower rate of hydrolysis and absorption, lactose has little influence on the blood-glucose level and therefore is less of a burden for diabetics than sucrose [204].
1.5 Utilization of Whey Proteins and Lactose

Wide applications of whey components are emerging in different areas based on their multi-functionalities.

1.5.1 Applications relating to Physicochemical Functionality

1.5.1.1 Uses of Whey Proteins in Food Industry

In general, whey protein is considered by the food industry to have excellent functional properties, though, in some cases, the functional properties of whey protein isolates produced by laboratory research have been overrated and overpromoted for specific applications [10]. Their functional properties have, however, encouraged attempts to use them in a great number of food products to replace other more traditional additives such as milk powder and egg albumen [13]. Few other protein materials possess properties similar to those of egg white, e.g., high sulphhydryl and disulphide contents, heat coagulability and foaming capability [205]. Table 1-11 gives a list of whey proteins and other functional proteins, and grades them according to their individual functional properties.

1.5.1.2 Uses of Lactose in Food and Pharmaceutical Industries

Lactose is used in many foods [59]. For soups, sauces, sauce binders, instant drinks, spice mixes and meat products, the primary reasons for the use of lactose are to reduce sweetness, to enhance aroma, to increase storage life, and to gain a price advantage over other ingredients.

Lactose is one of the best carriers for tablet-making [59]. A series of different lactose products is available to provide a range of granular distribution, free
flowability and bulk density to meet the growing demands of the pharmaceutical industry. These products include those that offer directly compressible properties, eliminating the need for tablet producers to use costly wet granulation processes.

1.5.2 Applications relating to Nutritional Functionality

1.5.2.1 Nutritional Uses of Whey Proteins

Whey protein have been recommended by Renner and El-Salam [101] for nutritional utilizations in the following areas:

1) The extraordinary supplementary effect of whey protein is significant when combined with other dietary proteins, for example, the PER of wheat protein could be increased from 0.8 to 2.5 in a wheat: WPC mixture.

2) There is increasing interest in the exploitation of the excellent nutritional status of whey proteins as food ingredients for high performance sportsmen.

3) WPCs are useful for the preparation of low-fat, high-protein diets, consequently they are used as components of slimming diets.

4) Because of the high PER values, WPC is an excellent food fortifier for those on restricted diets, particularly for geriatric and hospital uses. However, because Lg, an allergenic protein, is dominant in the whey-protein commercial preparations (WPCs and WPIs), there is a possible allergic reaction when using whey protein for nutritional purposes. An obvious method to avoid such reactions is to produce enzymatic hydrolysates of the total whey protein; for example, to hydrolyse Lg selectively by thermolysin digestion at 2000 atm [73, 74]. The fragmented or digested Lg no longer causes the allergenic reactions. From a nutritional view, these hydrolysates
offer the advantage that the essential amino acids of the whey proteins remain available [207].

1.5.3 Application relating to Physiological Functionality

1.5.3.1 General Aspects in Functional Food

The concept that “foods can have physiological functions beyond that of nutrition” was presented to the dairy industry at a Chicago Dairy Conference by Harper [112] in 1990. This oriental concept can be traced back to a Chinese materia medica book more than two thousands year ago, where the author claimed that “Food and medicine are same source (meaning food can play a role as medicine)”.

Combining oriental perception with occidental clinic of medicine, functional food (nutraceutical, therapeutic or biological food), pioneered by the Japanese, has been developed rapidly around the world. In Japan, legislators have seen the need for a regulatory category covering foods which have real, demonstrable medical benefits and have prepared legislation allowing health claims for these “physiologically functional foods” under controlled circumstances. The original Japanese definition of a functional food (or a specific health food) is one which [208]:

a) defends the body,
b) prevents disease,
c) cures disease,
d) regulates body rhythms, and
e) treats certain phenomena associated with aging.

Regarding the frontier between food and drugs, it should be noted that the above-given Japanese definition of a functional food cannot be used in Europe [208]
and USA [208] because the mere presentation of a product as ‘preventing and curing disease’ makes it a drug [208].

It is obvious that therapeutic or biological functions claimed by a functional food should be based on scientific or clinical support, and subject to legislative supervision. This development is expected to liberate over-restricted legislative definition between “food and drug” used in most nations, and also avoid abusing health claims and misleading customers.

The development of the functional food market and establishment of the relevant legislation in Japan will heavily influence north-east or south-east Asian countries where traditionally there are similar views on food, namely that medical benefits can be promoted by a specific health food. As a result, similar legislations will be introduced more easily into these countries than western countries.

The Australian food industry is keen to capitalise on growth in functional food export markets. The National Food Authority acknowledges that ‘regulatory reform may be needed to encourage innovative research into food product development’. The definition of functional foods is the subject of animated debate in Australia.

Many ingredients have currently been approved by Japanese authorities for use in “specific health food” products such as oligosaccharides, casein phosphopeptide, vitamins, polyunsaturated fatty acids, e.g. docosahexanoic acid (DHA), a brain-developing compound [209, 210].
Accordingly, *functional food* is so far concerned with the physiological functionality of a food or an ingredient. However, *functional foods* in a broad sense should be defined as a suggestion by this thesis:

**Functional foods refer to those foods and ingredients which are prepared by formulation and addition of purified or modified food components, and mainly used for their functional properties of regulation and characterization rather than their nutritional values for supporting life.**

1.5.3.2 Uses of Whey Proteins in Functional Food

Whey proteins including total whey protein, Igs, Lf etc. have sound physiological functions as discussed above which provide potential opportunity for utilization.

One of the more important applications is that of "humanized milk" in which Igs, Lf or Ly are added into bovine milk and infant formulas. Human milk is usually thought to be the model for infant formulas because it is the ideal food for infants. There is a difference in the composition and proportions of whey proteins and caseins found in bovine and human milk. Human milk contains higher Igs, Lf and Ly and lower casein and Lg. To "correct" these differences modern infant formulas are fortified with whey proteins (Ig, Lf or Ly) and the proportions of casein and Lg are reduced.

An immune milk produced by a joint venture called Stolle Milk Biologics International (SMBI), has been obtained from cows that have been regularly immunised with a proprietary polyvalent vaccine. Stolle immune milk containing much higher antibodies was marketed in Taiwan [209]. Thus, nutraceutical
applications for bovine antibodies may be expected in the near future. The potential market for this product is huge with one estimate of A$5 million for Australia, A$70 m in the United States, A$30 m in Japan, and A$60 m in Europe [121].

1.5.3.3 Thermal Properties of Whey Proteins

Since heat treatments of milk and whey are important steps in the production of dairy products, the loss of functionalities of whey proteins by the thermal denaturation is a major concern in application. The heat treatment of milk and whey may be usefully divided into a number of unit process operations, approximately according to the temperature range employed [9]:

- Thermization: 50-65°C
- Pasteurization: 65-75°C
- Functionalization: 75-90°C
- Sterilization: 90-140°C

The thermal properties of whey proteins have been extensively studied, and some thermal characteristics of the major whey protein components are summarized in Table 1-12. However, discrepant reports exist in the published literature because the effects of heat on whey proteins are greatly influenced by pH, ionic strength, the rate of heating, and, to a lesser extent, by protein and lactose concentrations [10].

The kinetic parameters for heat-induced denaturation of lactoferrin under different conditions were determined over a temperature range 72-85°C [211]. The thermal characteristics of Lf with different iron saturation and a mixture of Lg and 100% iron-saturated Lf are shown in Table 1-13. Lf is denatured more rapidly in its apo form than in the iron-saturated form. Both apo-Lf and iron-saturated Lf are more
heat-sensitive when treated in milk than in phosphate buffer. Values of change in 
enthalpy of activation of Lf denaturation are high which indicates that a large number 
of bonds are broken. The association of Lf with Lg does not significantly influence 
the change in enthalpy of activation of Lf denaturation. The study on denaturation of 
bovine milk Lf has concluded [211] that the widely used pasteurization treatment at 
72–74°C for 15 s had practically no effect on Lf structure.

Thermal stability of bovine Igs in model systems and in commercially 
processed milk products has also been investigated recently [212]. Bovine serum IgG 
dissolved in either phosphate buffered saline (PBS), boiled milk or ultra high 
temperature (UHT) sterilized milk was heated at temperatures ranging from 62.7°C 
to 80°C. The D-values (time for a 90% reduction in IgG activity) ranged from 90, 
200 and 170 s at 80°C to 25.5, 27.2 and 32.8 min at 72°C for IgG in PBS, boiled 
milk and UHT milk, respectively. These results suggest slightly greater thermal 
stability of IgG in milk than in phosphate buffer. IgG content was not changed after 
30 min at 62.7°C. These findings in model systems suggest that commercial 
pasteurization processes should not result in complete destruction of IgG. IgG 
contents and specific antibody activity against lipopolysaccharide fractions of five 
bacteria were determined for several commercially processed milk products [212]. 
Pasteurized milks, reconstituted skim milk powder and whey from Cheddar cheese 
production all showed high levels of IgG and specific antibody activity. However. 
UHT sterilized milk had little or no IgG.

1.5.3.4 Uses of Lactose
Lactose has been used for biological purpose in chicken feeds for growth promotion. Addition of lactose 0.1-3.0% (w/w) in chicken feeds has resulted in an increase of body weight and reduction of body fats of the chicken [199].

Lactose is also used in the production of penicillin [59]. The use of lactose in fermentation broths offers advantages owing to the delayed decomposition of the lactose [213] over glucose, leading to catabolite repression.

1.6 Conclusion: Total Whey Utilization

With an extensive review of whey components and their processing, a strategy of Total Whey Utilization has been proposed as shown in Fig.1-6. The "Total Whey Utilization" is based on the fact that research activities in whey fractionation have shifted to the isolation of the finer components, such as β-lactoglobulin, lactoferrin, glycomacropeptide and immunoglobulins, and to the issue of the disposal of whey or permeate as wastewater. It has been well known from the reviews that individual protein components in whey have different functionalities. Best performance of a whey protein in functionality is based on the elimination of antagonistic effects of other whey proteins or impurities. Although the reviews have indicated that many technologies were investigated for the isolation of individual whey protein, no one has approached a system for the total utilization of whey solids by processes which fit together in a complementary fashion. In addition, the mother liquor after lactose crystallization has a heavy load of pollution.
This thesis will focus on the development of novel separation methods which would be suitable for the *Total Whey Utilization* process. In addition, the mechanisms of the separation methods have also been investigated. The studies include pretreatment, interaction of Ca\(^{2+}\) and lactoferrin, fractionation of immunoglobulins and glycomacroppeptide, and separation of lactose. When the *Total Whey Utilization* process is successful, this one scheme allows manufacture of WPC and lactose as well as Lf, Ig, GMP without any waste.
1.7 References


APPENDIX 1-1
TABLES IN CHAPTER 1
<table>
<thead>
<tr>
<th>Composition</th>
<th>SWEET WHEY</th>
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<td>Camemb.</td>
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<tr>
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Table 1-1 Composition of Sweet and Acid Whey (Pearce [2])

* N/A: not available

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<td>Whey&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>17,928</td>
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Table 1-2 Whey and Lactose Imports/Exports for 1989-1993 (ABS [8])

<sup>a</sup> Whey: Whey preserved, concentrated or sweetened.
<sup>b</sup> Whey products: Products consisting of natural milk constituent not elsewhere specified.
<sup>c</sup> Lactose: Lactose and lactose syrup.
<table>
<thead>
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<th>Proportion of Total Whey protein (%)</th>
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<td>C-5</td>
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<tr>
<td>(*β-CN-5P(1-105))</td>
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<td>(*β-CN-5P(1-107))</td>
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<td>C-8-fast</td>
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<td>Other proteins</td>
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<td>5&lt;sup&gt;l&lt;/sup&gt;Lip</td>
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Table 1-3 Compositions of Proteins in Whey<sup>a</sup>


<sup>b</sup>Acid Whey; <sup>c</sup>Sweet Whey; <sup>d</sup>Colostral Whey; <sup>e</sup>Conversion from the Chemical Composition of Colostrum [216]; <sup>f</sup>Lipoprotein; *Recommended Nomenclature of the PP by Eigel [9].
<table>
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<tr>
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<td>6-220</td>
<td>79,82</td>
<td>5-11</td>
<td>12-16</td>
<td>40-43</td>
</tr>
<tr>
<td>28-111</td>
<td>84,87</td>
<td>23-34</td>
<td>17-2</td>
<td>47-50</td>
</tr>
<tr>
<td>61-77</td>
<td>88</td>
<td>86-99</td>
<td>76-82</td>
<td></td>
</tr>
<tr>
<td>73-91</td>
<td>105-109</td>
<td>101-104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>115-119</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1-4: Location of Three-Dimensional Structure Elements in the Sequence of Baboon α-Lactalbumin\(^a\).

\(^a\) Date from Brew & Grobler [15], Stuart et al. [16], Kinsella & Whitehead [10].

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Nominal Concentration (mg/ml)</th>
<th>MW(^a) (Daltons)</th>
<th>pH(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>0.1</td>
<td>~420,000</td>
<td>~11</td>
</tr>
<tr>
<td>IgG(_1)</td>
<td>0.59</td>
<td>163,000</td>
<td>5.5-6.8</td>
</tr>
<tr>
<td>IgG(_2)</td>
<td>0.2</td>
<td>150,000</td>
<td>7.5-8.3</td>
</tr>
<tr>
<td>IgM</td>
<td>0.05</td>
<td>~1000,000</td>
<td>N/A</td>
</tr>
<tr>
<td>Non-Immunoglobulins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>~0.03</td>
<td>78,000</td>
<td>~8.0</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.02-0.35</td>
<td>76,000</td>
<td>~8.0</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.1-0.2(^c)</td>
<td>18,000</td>
<td>9.5</td>
</tr>
<tr>
<td>Xanthine Oxidase</td>
<td>0.035</td>
<td>155,000</td>
<td>6.9-7.6</td>
</tr>
<tr>
<td>Sulphydryl Oxidase</td>
<td>0.01</td>
<td>1000,000</td>
<td></td>
</tr>
<tr>
<td>Superoxide Dismutase</td>
<td>0.15-2.5(^c)</td>
<td>32,000</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-5: Important Biologically Functional Proteins in Whey

\(^a\) Molecular Weight
\(^b\) Isoelectric Point
\(^c\) µg/ml

Data from Björck [33]; Kinsella and Whitehead [10]; Farkye [35].
<table>
<thead>
<tr>
<th>Functional property</th>
<th>Mode of action</th>
<th>Food systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>Protein solvation</td>
<td>Beverages</td>
</tr>
<tr>
<td>Water adsorption and binding</td>
<td>Hydrogen bonding, Entrapment of water</td>
<td>Meats, sausages, cakes, breads</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Thickening, water binding</td>
<td>Soups, gravies, salad dressing</td>
</tr>
<tr>
<td>Gelation</td>
<td>Protein matrix formation and setting</td>
<td>Meats, curds, baked goods, pasta products</td>
</tr>
<tr>
<td>Elasticity</td>
<td>Hydrophobic bonding in gluten, disulfide links in gel</td>
<td>Meats, bakery</td>
</tr>
<tr>
<td>Emulsification</td>
<td>Formation and stabilization of fat emulsions</td>
<td>Sausages, salad dressing, coffee whitener, soup, cakes infant formula</td>
</tr>
<tr>
<td>Fat adsorption</td>
<td>Binding of free</td>
<td>Sausages, doughnuts</td>
</tr>
<tr>
<td>Foaming</td>
<td>Forms stable film to entrap gas</td>
<td>Chiffon desserts, cakes, whipped toppings</td>
</tr>
</tbody>
</table>

Table 1-6 Typical Functional Properties of Proteins in Model Food Systems (Mangino [103])
<table>
<thead>
<tr>
<th>Essential Amino Acids</th>
<th>Reference Protein, FAO</th>
<th>Whole Egg</th>
<th>Milk Protein</th>
<th>Whey Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Try</td>
<td>1.0</td>
<td>1.5</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Phe + Tyr</td>
<td>6.0</td>
<td>10.5</td>
<td>10.5</td>
<td>7.3</td>
</tr>
<tr>
<td>Leu</td>
<td>7.0</td>
<td>9.1</td>
<td>10.4</td>
<td>11.1</td>
</tr>
<tr>
<td>Ile</td>
<td>4.0</td>
<td>6.7</td>
<td>6.4</td>
<td>6.8</td>
</tr>
<tr>
<td>Thr</td>
<td>4.0</td>
<td>5.1</td>
<td>5.1</td>
<td>8.0</td>
</tr>
<tr>
<td>Met + Cys</td>
<td>3.5</td>
<td>5.9</td>
<td>3.6</td>
<td>4.8</td>
</tr>
<tr>
<td>Lys</td>
<td>5.5</td>
<td>6.9</td>
<td>8.3</td>
<td>9.9</td>
</tr>
<tr>
<td>Val</td>
<td>5.0</td>
<td>7.5</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Total</td>
<td>36.0</td>
<td>53.2</td>
<td>52.5</td>
<td>56.8</td>
</tr>
</tbody>
</table>

**Table 1-7 Concentrations of Essential Amino Acids in Food Proteins (Renner [108])**

<table>
<thead>
<tr>
<th>Food Protein</th>
<th>BV(^a)</th>
<th>PER(^b)</th>
<th>NPU(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Egg</td>
<td>100</td>
<td>3.8</td>
<td>94</td>
</tr>
<tr>
<td>Bovine Milk</td>
<td>91</td>
<td>3.1</td>
<td>82</td>
</tr>
<tr>
<td>Casein</td>
<td>77</td>
<td>2.9</td>
<td>76</td>
</tr>
<tr>
<td>Whey Protein</td>
<td>104</td>
<td>3.6</td>
<td>92</td>
</tr>
<tr>
<td>Beef</td>
<td>80</td>
<td>2.9</td>
<td>73</td>
</tr>
<tr>
<td>Soy</td>
<td>74</td>
<td>2.1</td>
<td>61</td>
</tr>
<tr>
<td>Potato</td>
<td>71</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rice</td>
<td>59</td>
<td>2.0</td>
<td>57</td>
</tr>
<tr>
<td>Wheat</td>
<td>54</td>
<td>1.5</td>
<td>41</td>
</tr>
<tr>
<td>Beans</td>
<td>49</td>
<td>1.4</td>
<td>3.9</td>
</tr>
</tbody>
</table>

**Table 1-8. Nutritional Value of Proteins (Renner and El-salam [101])**

\(^a\)BV = biological value, \(^b\)PER = protein efficiency ratio
\(^c\)NPU = net protein utilization
Table 1-9 Physiologically Functional Proteins or Protein-base Factors in Whey (Harper [112])

<table>
<thead>
<tr>
<th>Lactoferrin</th>
<th>Lactoperoxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulins</td>
<td>Bifidus growth factors</td>
</tr>
<tr>
<td>Intestinal regulation factor</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Hormones</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-10 Biological Functionality of Lactoferrin

<table>
<thead>
<tr>
<th>Antimicrobial Activity</th>
<th>Growth Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-cancer</td>
<td>Anti-oxidation</td>
</tr>
<tr>
<td>Immunity</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Inhibition of Cholesterol Accumulation</td>
<td></td>
</tr>
<tr>
<td>Diversified Binding Properties</td>
<td></td>
</tr>
<tr>
<td>Functional Property</td>
<td>WPC&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Solubility</td>
<td>3-4</td>
</tr>
<tr>
<td>Emulsification</td>
<td>3</td>
</tr>
<tr>
<td>Foaming</td>
<td>0-3</td>
</tr>
<tr>
<td>Heat setting</td>
<td>2-4</td>
</tr>
<tr>
<td>Water holding</td>
<td>1</td>
</tr>
<tr>
<td>Blandness</td>
<td>0-3</td>
</tr>
</tbody>
</table>

Table 1-11 Functional Properties of Commonly used Food Proteins<sup>a</sup>

<sup>a</sup> Data from West 1984 [206]; <sup>b</sup> Whey Protein Concentrate; <sup>c</sup> Whey protein Isolate;
<sup>d</sup> Values for dried egg albumen in brackets; <sup>e</sup> Unless hydrolysed
0 = very poor, 4 = excellent (where a range is given, this means that different quality products are available, depending on how they have been processed)

<table>
<thead>
<tr>
<th></th>
<th>$T_d$</th>
<th>$T_{tr}$</th>
<th>DH (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Lactalbumin</td>
<td>62</td>
<td>68</td>
<td>253</td>
</tr>
<tr>
<td>$\beta$-Lactoglobulin</td>
<td>78</td>
<td>83</td>
<td>311</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>64</td>
<td>70</td>
<td>803</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>72</td>
<td>89</td>
<td>500</td>
</tr>
<tr>
<td>Whey proteins</td>
<td>62-78</td>
<td>68-83</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 1-12 Thermal Denaturation Temperature (°C) and Enthalpies of Whey Proteins<sup>a,b</sup>

<sup>a</sup> Collected from Kinsella and Whitehead [10], data after deWit [215]
<sup>b</sup> $T_d$, Initial denaturation temperature; $T_{tr}$, temperature at maximum peak on thermogram peak temperature in differential scanning calorimetry; DH is the enthalpy of denaturation. N/A: not available
<table>
<thead>
<tr>
<th>Protein</th>
<th>$T_{D1}$ (°C)</th>
<th>$T_{D2}$ (°C)</th>
<th>DH (J/g)</th>
<th>DH (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-lactoferrin</td>
<td>60.8</td>
<td></td>
<td>8.6</td>
<td>684</td>
</tr>
<tr>
<td>Lactoferrin sat. 30%</td>
<td>69.6</td>
<td>86</td>
<td>17.6</td>
<td>1,410</td>
</tr>
<tr>
<td>Lactoferrin sat. 100%</td>
<td>74</td>
<td>86.5</td>
<td>21.0</td>
<td>1,600</td>
</tr>
<tr>
<td>Mixture of Lg + Lf (1:1)</td>
<td>74.3</td>
<td>86.6</td>
<td>26.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-13 Denaturation Temperatures and Enthalpies of Denaturation for Lactoferrin with Different Degrees of Iron Saturation and a Mixture of β-Lactoglobulin\textsuperscript{a,b}

\textsuperscript{a} Data after Sanchez et al. [211]

\textsuperscript{b} $T_D$, denaturation temperature; The footnotes of 1, 2 represent first and secondary peak temperature on the thermogram of differential scanning calorimetry, respectively; DH is the enthalpy of denaturation.
APPENDIX 1-2
FIGURES IN CHAPTER 1
Fig.1-1 Amino Acid Sequence of $\kappa$-casein B (Sleigh [27])

$\downarrow$ Cleavage of the rennin-sensitive bond at F$^{105}$-M$^{106}$ splits $\kappa$-casein B into para $\kappa$-casein (E$^1$-F$^{105}$) and GMP (M$^{106}$-V$^{169}$)
T$^{131}$ (underlined boldface) is carbohydrate-binding site.

Fig.1-2 Schematic of IgG molecule [28]
Antigen binding occurs in the $\chi_{H} / \chi_{L}$ domain interface. Biological effector functions reside in the C$\gamma_1$ to C$\gamma_3$ domain. The hinge region accounts for the segmental flexibility of the molecule. Note that the interchain disulfide bonding pattern is characteristic of each subclass of IgG (e.g., IgG3 has two inter-heavy chain disulfide bond bridges the light chain to a cysteine residue that is closer to the hinge region than the other subclasses).
Fig. 1-3 Primary Structural Features of Bovine Lactoferrin

Single-letter code is used to indicate the amino acid sequence (according to Pierce et al. [40]). The bactericidal domain of the N-lobe (residues 17-41) and corresponding region of the C-lobe (residues 356-376) are bold-faced. Putative iron binding residues are bracketed and potential glycosylation sites are marked with asterisks.

Fig. 1-4 Schematic Representation of the Structural Changes Associated with Binding and Release of Iron by the Lactoferrin [214]

For the unliganded form (left) an equilibrium is presumed to exist between open and closed states. Binding is to the open form, which in the ligand is bound to one domain only; domain closure then gives the fully-closed liganded form (lower right). • represents iron ion.
A: The folding pattern for the lactoferrin molecule. Helices are shown as cylinders, β-strands as arrows, iron atoms ⋆, probable anion site o, disulphide – – – –, and carbohydrate attachment sites ★ (labelled L, lactoferrin; T, serum transferrin; O, ovotransferrin; and M, potential sites in melanotransferrin). The N-terminal half (N-lobe) is at top, the C-terminal half (C-lobe) at bottom; their relative orientations, related by a twofold screw axis, are shown in the inset. The two domains in each lobe are labelled 1 and 2. A region where hydrophobic interactions between the two lobes are made is indicated (H). Helices labelled are the connecting helix (A) and C-terminal helix (B). Note how the latter forms an extra connection between the C-lobe domains, not present in the N-lobe.

B: The iron site in the lobe of lactoferrin. Numbering is for the N-lobe (equivalent residues in the C-lobe are Asp407, His609, Tyr540, Arg477). The location of density attributable to the anion (and / or co-ordinated water molecules) is shown.

Fig. 1-5 Schematic Diagram of the Lactoferrin Molecule and the Iron Site in the Lobe [44]
Acid, Sweet or Colostral Whey

Clarification

Pretreatment (FRL/UWS method) (Chapter 3)

Clear Supernatant

UF Fractionation 10 kDa cut-off

Retentate

Fractionation by Ion exchange or UF 100 kDa (Chapter 5)

Lg-Rich Igs-Rich

Ion Exchange

GMP (Chapter 5)

Casein Fines, Fat

Colloidal Precipitates containing Lactoferrin

WPC

Permeate

Evaporation and Crystallization

Mother Liquor

Separation (Chapter 6)

Impurities Lactose Fraction

Lg = Lactoglobulin; Igs = Immunoglobulins; WPC = Whey Protein Concentrate.

Fig. 6-1 Total Whey Utilization
Chapter 2 Literature Review 2:
Theoretical Considerations of Methodologies in This Thesis

Abstract

This chapter reviews some important and advanced techniques which have been used in this thesis such as Affinity Precipitation, Membrane Technology, Fourier Transform Infrared Spectroscopy, Enzyme-linked Immunoassay, and Chromatographic Separation. The discussion focuses on the theoretical basis, and the applications of these techniques.
2.1 Colloidal Aggregation

2.1.1 Milk and Whey —— the Colloid System

The simplest colloid consists of a dispersed phase —— particles or droplets or micelles approximately 1 nm to 1 mm in size —— suspended in a continuous phase which is the dispersion medium [1].

Many foods such as milk [2] are colloidal systems, containing particles of various kinds. Milk contains three major sorts of colloidal particle, i.e. fat globules, casein micelles and lipoprotein particles [3].

The casein micelle is the most important colloid in the milk. Its structure has been proposed by several models among which two models, the network model and the subunit model, are broadly in line with the experimental facts.

Assuming a mode of association of the caseins, Garnier and Ribadeau-Dumas [2] proposed a model which depicts the casein micelle as a three-dimensional porous network of protein aggregates. In this network, three κ-casein molecules act as nodes, and branches consist of αs- and β-caseins. In this model, calcium phosphate does not have an essential role though it was suggested to bind to the casein network.

A model to in which the casein micelle is composed of subunits of variable composition was proposed by Slattery and Evard [2]. Some of subunits in this model consist of αs- and β-caseins, while the rest also contain κ-casein. The hydrophobic regions of αs- and β-caseins are responsible for the mutual binding of the subunits in the micelle, whereas the hydrophilic κ-casein areas are exposed to the solvent. In
another sub-unit model proposed by Schmidt and Payens [2] (shown in Fig.2-1 [3]), it is suggested that casein micelles consist of a large number of ‘sub-micelles’ held together by colloidal calcium phosphate in an open, disordered structure (Fig.2-1a). The sub-micelles contain 25-30 molecules (\( \sim 6 \times 10^5 \) daltons) of \( \alpha_{\text{s1}}, \alpha_{\text{s2}}, \beta \) and \( \kappa \)-casein held together by hydrophobic interaction (some ionic bonding probably also occurs between \( \alpha_{\text{s1}} \)-and \( \kappa \)-casein). Sub-micelles resemble conventional (soap) micelles insofar as hydrophobic groups concentrate at the centre and polar groups at the surface. In the ‘coat-core’ representation (Fig.2-1b), a ‘coat’ of \( \kappa \)-casein envelopes a ‘core’ of \( \alpha_{\text{s1}}, \alpha_{\text{s2}}, \beta \) and \( \kappa \)-casein. Later, some extensions of the subunit model have been proposed by different workers [2].

Whey, obtained by acidification or enzyme-clotting, appears cloudy although fat has been removed. It is a relatively dilute colloidal solution containing residual caseins, calcium phosphate, and other substances.

2.1.2 Colloidal Aggregation

A colloidal dispersion is stable, if, over a certain period of time, there is little aggregation of the particles [1].

The classic view of coagulation (aggregation) as a diffusion-controlled phenomenon goes back to von Smoluchowski in 1916 [6]. The principal physical idea is that, due to Brownian motion, pairs of particles in a dilute dispersion diffuse towards each other and, on making contact, one of the particles is deemed to ‘disappear into a sink’ [6].
Fig.2-2 summarizes the interparticle region in various kinds of aggregates including colloidal "attraction", depletion flocculation, bridging flocculation, cross-linking, bridging by particles, and neck formation.

2.1.2.1 Interaction Forces of Aggregation [4]

Aggregation of the particles depends primarily on the interaction forces between the particles [4].

The Deryagin-Landau-Verwey-Overbeek (DLVO) theory considers that there are interaction forces of colloidal aggregation, namely the electrostatic repulsion and van der Waals attractions. For two identical homogeneous spheres of radius \( a \), the free energy \( G \) which is needed to bring two particles from infinite distance apart to a close distance between their surfaces, \( h \), can be written as follows:

\[
G_R \approx 4.3 \times 10^{-9} a \Psi_0^2 \ln (1 + e^{-kh})
\]

(2-1)

and

\[
G_A \approx -Aa/12h
\]

(2-2)

\( G_R \) is the repulsive free energy, and \( G_A \) is the attractive free energy. If expressed as SI units, \( \Psi_0 \) is the surface potential of the particles; \( k \) is the Debye-Huckel parameter; \( A \) is the Hamaker constant; \( h \) is the surface separation distance of two identical particles.

The DLVO theory has been fairly successful in predicting the aggregation stability of inorganic colloids, though it is rarely exact for food colloids [4]. However, the theory can be used to qualitatively describe the aggregation of food colloids fairly well. For example [4], lowering the surface potential — e.g. by altering the pH — or increasing the ionic strength diminishes the electrostatic repulsion and thereby promotes aggregation.
2.1.2.2. Measurement of Particle Size by Light-scattering and Turbidity

When light is directed at a dilute colloidal system in a glass container, most of it passes through undisturbed, but some is scattered and some is absorbed.

The intensity of light scattered at a certain angle to an incident monochromatic beam is dependent on several factors: (i) the wavelength and polarization of the incident beam, (ii) the difference in refractive index between dispersed and continuous phases, (iii) the sizes and shapes of the particles, and (iv) the concentration and structure of the dispersion.

The intensity of scattered light may be measured directly and used to estimate particle size, but it is necessary for the volume fraction of the dispersed phase to be very low so as to minimize multiple scattering and interparticle interference [3]. Alternatively the intensity of scattered light may be measured indirectly by recording the diminution in intensity of a light beam on passing through a colloidal dispersion. The amount of light absorbed by a dilute solution or dispersion of the colloid is expressed as optical density (O.D) [5]

\[ O.D = \log_{10}(I/I_0) = -\log_{10}T \]  

(2-3)

where \( I_0 \) is the incident light intensity, \( I \) is the transmitted light intensity, and \( T \) is the optical transmittance. The turbidity (\( \tau \)) of the colloidal solution is then:

\[ \tau = 1/L \log_e 1/T \]  

(2-4)

where \( L \) is the optical path length.

In a light-scattering solution, the turbidity (\( \tau \)) is related to molecular weight by [7, 8]:

\[ t = H \cdot Q \cdot M_w \cdot c \]  

(2-5)
where $H$ is an optical constant, $Q$ is a correction factor, and $c$ is the concentration. If the particles are of unequal size, $M_w$ is the weight-average molecular weight of the particles. As turbidity is proportional to $M_w$ of the particles, measurement of turbidity allows detection of the growth of the particles with respect to the behaviour of colloidal aggregates.

For a von Smoluchowski type of reaction (fast coagulation), the growth of $M_w$ for aggregating material with time is given by [7]:

$$M_w(t) = M_w(0) (1 + k_c c_0 t)$$  \hspace{1cm} (2-6)

where $k_c$ is the rate constant for coagulation, $c_0$ is the initial molar concentration of aggregating material, $t$ is the time and $M_w(0)$ the weight-average molecular weight at $t = 0$.

2.2 Affinity Precipitation

Affinity precipitation is a specific purification technique based on the interaction between a target protein and a ligand which binds to the protein in a specific manner [9]. Affinity precipitation includes two processes, i.e. formation of the protein-ligand complex and precipitation of the complex. So this technique is closely related to affinity chromatography and immunoprecipitation [10].

2.2.1 Choices of Ligand

In the chemistry of coordination compounds, a ligand is defined as a species that is capable of donating an electron pair to a central ion [11]. In affinity chromatography, a ligand, on the other hand, is a molecule able to interact or bind with the protein [12]. For the affinity chromatography and precipitation, choice of an appropriate ligand is
the first step. The factors to consider when selecting a ligand for protein purification are discussed as follows [12]:

(i) *Specificity*. Ideally, the ligand should bind only to the protein to be purified.

(ii) *Reversibility*. The ligand should form a reversible complex with the protein to be purified such that the complex is resistant to the composition of the feedstream and washing buffers but is easily dissociable without requiring denaturing conditions for elution.

(iii) *Stability*. The ligand should be stable to the conditions to be used for immobilization as well as the conditions of use.

(iv) *Size*. The ligand should be large enough to allow it to contain several groups able to interact with the protein resulting in sufficient stereoselectivity and affinity.

(v) *Affinity*. The interaction of a protein P and a ligand L can be described by the equation:

\[ P + L \rightleftharpoons PL \]  

(2-7)

where the equilibrium dissociation constant, \( K_{\text{dis}} \), is defined by:

\[ K_{\text{dis}} = \frac{[P] [L]}{[PL]} \]  

(2-8)

A simple rearrangement gives:

\[ \frac{[L]}{K_{\text{dis}}} = \frac{[PL]}{[P]} \]  

(2-9)

which implies that for substantial adsorption of the protein from solution (e.g. \([PL] : [P] = 95:5\)) the value of \( K_{\text{dis}} \) must be about two orders of magnitude less than the concentration of immobilized ligand. As a general rule affinity techniques operate well between \( K_{\text{dis}} = 10^{-4} \) M and \( 10^{-8} \) M.

2.2.2 Mechanism of Affinity Precipitation
Affinity precipitation of protein can occur via two general mechanisms [9], shown schematically in Fig. 2-3.

The first mechanism involves forming networks of the multivalent protein molecules by cross-linking them with a bis-ligand or polyligands. Under proper conditions these networks will grow large enough to become insoluble and form a precipitate. This mechanism is referred to as either affinity precipitation with homobifunctional ligands or primary-effect precipitation. This method is similar to antigen-antibody precipitation, a major analogy being that the ligand-to-binding-site ratio must be near unity for maximal precipitation to occur.

In the second mechanism, complexes are formed between target protein molecules and ligands which are bound to water-soluble polymers, and the complexes are precipitated either by reducing the solubility of the polymer though changes in pH or temperature or by cross-linking the polymer with a poly-binding protein. This mechanism is referred to as either affinity precipitation with hetero-bifunctional ligands or secondary-effect precipitation.

2.2.3 Calcium Phosphate Adsorbent

There are two kinds of calcium phosphate [13], i.e. brushite or calcium phosphate gel (CaHPO₄·2H₂O) and crystalline calcium phosphate or hydroxylapatite \{Ca₁₀(PO₄)₆(OH)₂\}. Hydroxylapatite (HA) is prepared by boiling brushite with sodium hydroxide or ammonia. Calcium phosphate gels have been used in protein purification for some time [12]. However, it was not until the development of hydroxylapatite that the flow characteristics of this adsorbent were improved sufficiently to allow its successful use in column chromatography.
HA absorbents are amphoteric and the isoelectric points of different HA preparations have been found to range from 6.5 to 10.2 [13]. The mechanism of protein adsorption onto HA is thought involve both Ca$^{2+}$ and PO$_4^{3-}$ groups on the calcium phosphate surface [12].

2.2.4 Metal Affinity Precipitation

Recently, metal affinity precipitation [14] of proteins was demonstrated with bishelates of Cu (II) as macroligands. Using the known affinity between Cu (II) and metal coordinating residues on the protein surfaces, two cupric cation chelated by molecules of iminodiacetic acid (IDA) immobilized onto a single spacer molecule of polyethylene glycol (PEG) or chelated by a molecule of ethylene glycol bis(b-aminoethyl ether)-N,N'-tetraactic acid (EGTA) will result in bis-metal affinity ligands to crosslink and precipitate proteins that contain multiple metal coordination sites.

2.3 Membrane Technology

Membranes for use in large-scale commercial processes were not developed until the invention of the asymmetric membrane in the late 1950s [15]. Membrane technology is still rapidly evolving and new processes are presently undergoing development [15].

2.3.1 Classification of Membrane Technology

So far, a rough classification of membrane types can be made on the basis of the (molecular) size of the product to be separated, as is shown in Fig.2-4.

2.3.1.1 Microfiltration, Ultrafiltration and Nanofiltration
Microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF) involve the pressure-driven liquid filtration through porous membranes with a narrow range of pore size. MF, UF and NF may be distinguished by the size of the particle or molecule the membrane is capable of retaining or passing. By contrast, reverse osmosis (RO) and pervaporation (PV) make use of tight, dense membranes [15].

MF is used to remove suspended solids from liquid streams, on the other hand, UF and NF are involved in fractionation of the compounds with different molecular weight (Fig.2-4). Nanofiltration [16] is a relatively new niche in the spectrum of sieving membranes, which lies between UF and RO. The lower limit for molecular weight cutoff of UF is normally considered to be 5 kDa, whereas the molecular weight cutoff of RO is smaller than the size of inorganic ions. A UF membrane has pores, whereas the active part of a RO membrane has no pores other than molecular interstices. The active part of a NF membrane contains detectable pores and, like RO, relies on pressure to solvent through the membrane, but with high flux and greater discrimination of molecules below 5 kDa.

2.3.1.2 Reverse Osmosis and Pervaporation

As mentioned above, reverse osmosis (RO) and pervaporation (PV) make use of tight, dense membranes and are able to separate species that have comparable sizes, such as sodium chloride and water [15]. Pervaporation [17] is a membrane process at the feed side or upstream side and where the permeate is removed as a vapour at the downstream side by applying a low partial pressure. This can be achieved either by creating a vacuum or by using a carrier gas. Such the driving force in PV could be a
different aspect of RO. The driving force of RO is similar to MF, UF and NF, where the pressure produced by a pump is used.

2.3.1.3 Electro dialysis and Bipolar Membranes

In electrodialysis, charged (ion exchange) membranes are used to separate molecules or ions in an electrical field on the basis of differences in charge and transport velocity through the membrane. In an electrodialysis cell a number of cation and anion exchange membranes are placed between an anode and a cathode. When a gradient of electromotive force is applied between the electrodes, positively charged ions migrate through the cation exchange membrane while the negatively charged ions migrate through the positively charged anion exchange membrane. Another recent breakthrough is the so-called bipolar membrane [15]. Bipolar membranes can be used in water-splitting processes (\( \text{H}_2\text{O} \rightarrow \text{H}^+ + \text{OH}^- \)), and offer possibilities for the recovery of organic and amino acids [15]. An example of applying a bipolar membrane in combination with electrodialysis is the production of lactic acid from a sodium lactate solution, where the negatively charged lactate migrates through the anion exchange membrane and recombines with the \( \text{H}^+ \) ion generated by the bipolar membrane [15].

2.3.2 Decisive Engineering Parameters

Although UF process is used as the model for the parameters discussed below, in principle, the effects generally occur in every membrane process but are dominant in MF, UF, and RO.

2.3.2.1 Membrane Flux [18]
The fluid flow through the microporous membrane can be described by the Hagen-Poiseuille law for streamline flow through pipes:

\[ J = \varepsilon r^2 P / 8\eta \Delta x \quad (2-10) \]

where \( J \) is the flow rate through the membrane (flux in liters per unit area per unit time); \( r \) is the channel radius (mean pore radius); \( P \) is the applied pressure; \( \eta \) is the viscosity of fluid; \( x \) is membrane thickness; and \( \varepsilon \) is the surface porosity of the membrane.

For any membrane, the flux can be predicted from the following simple equation:

\[ J = K_m \Delta P - \Delta \pi \quad (2-11) \]

where \( K_m \) is a constant; \( \Delta P \) is the pressure difference between the upstream membrane surface and permeate side; and \( \Delta \pi \) is the difference in osmotic pressure of the solution on both sides of the molecule.

In the UF, \( \Delta \pi \) is negligible. Thus the equation can be written as follow:

\[ J = K_m \Delta P \quad (2-12) \]

2.3.2.2 Membrane Flux involving Concentration Polarization

When a solution is ultrafiltered, the solutes are brought to the membrane surface by convective transport. This results in building up a concentrated solute at the membrane surface. At steady state, the transport of solutes to the membrane surface must be balanced by an equal and opposite transport of solutes by diffusion from the membrane surface generating a concentration gradient of solutes from the membrane surface to the bulk stream. This phenomenon is known as concentration polarization [18].

Accordingly, the flux can be obtained from the following equation:

\[ J = \Delta P / R_m + R_\alpha \quad (2-13) \]
where $\Delta P$ is the transmembrane pressure drop

$R_m$ is the resistance of the gel layer

$R_\alpha$ is the resistance of the membrane

### 2.3.2.3 Fouling

Flux decrease due to gel-layer formation by concentration polarization should not be confused with the effect of membrane fouling which can also be caused by other phenomena, such as the adsorption of proteins and plugging of pores (Fig.2-5). Hence, a reversible and direct decline in flux across the membrane might be defined as concentration polarization, whereas irreversible and long-term decline in the flux are termed membrane fouling [15].

### 2.3.2.4 Rejection Coefficient and Permeability[18]

The rejection coefficient or permeability is the quantitative measure of the characteristic ability of a membrane to retain a solute species under specific operation parameters. It is calculated as

$$R_r = \frac{C_p}{C_r} = 1 - \frac{C_p}{C_r}$$  \hspace{1cm} (2-14)

where $R_r$ is the coefficient of rejection

$C_p$ is the solute concentration through the membrane (permeate)

$C_r$ is the solute concentration upstream of the membrane (retentate)

The equation can also be written as

$$R_p = \frac{C_p}{C_r}$$  \hspace{1cm} (2-15)

where $R_p$ is the permeability

### 2.4. Fourier Transform Infrared (FTIR) Spectroscopy
2.4.1 Instrumentation and Computation [19,20]

Infrared spectroscopy is one of the earliest experimental methods to be recognised as useful for estimating the secondary structure of proteins. In the past, the practical usefulness of the method with conventional (dispersive) infrared spectrometers was, however, severely limited by such factors as the low sensitivity of the instruments, interfering absorptions from the surrounding medium and, most importantly, by difficulties in extracting the structural information contained in the less conformation-sensitive infrared bands. Those obstacles have been largely overcome by the development of computerized FTIR instruments. This has improved the signal-to-noise ratio and also allowed extensive data manipulation.

FTIR spectroscopy relies upon the principles of interferometry and Fourier transformation for its speed and sensitivity. Conventional (dispersive) infrared spectrometers irradiate the sample sequentially with a narrow range of wavelengths produced by monochromation of a polychromatic beam until the entire spectral range has been scanned. FTIR spectroscopy on other hand irradiates the sample with all wavelengths simultaneously. This is achieved by directing a polychromatic beam onto a beam splitter, which directs the beam onto mirrors at angles to each other. A schematic diagram of a FTIR spectrometer is shown in Fig.2-6. The advent of microcomputer controlled spectrometers and the development of the Fourier transform data collection and manipulation methods associated with FTIR spectroscopy have greatly increased the areas of application of the technique.

2.4.2 Resolution Enhancement of Infrared Spectra —— Deconvolution and Derivative Techniques
As mentioned above, the greatest difficulty for conventional infrared spectrometer is that of extracting the structural information contained in the conformation-sensitive infrared bands. It reflects the fact that the conformation-sensitive amide bands of proteins are composites which consist of overlapping component-bands originating from different structures, such as α-helices, β-strands, turns and non-ordered polypeptide fragments.

FTIR has been given a significant step forward in the infrared spectroscopic analysis of proteins by using recently developed computational procedures for resolution enhancement methods which allow the decomposition of the complex amide bands into their underlying components. This new methodology not only enriches the qualitative interpretation of infrared spectra, but also provides a basis for the quantitative estimation of protein secondary structure. Resolution-enhanced infrared spectra of proteins were first obtained and analyzed by Susi and Byler in 1983 [21], and subsequently the method has grown in popularity. Many spectral absorption bands are complex, resulting from the overlap of two or more bands whose halfwidths are such that they cannot be separated by increasing instrumental resolution. Any single infrared absorption band can be expressed as the convolution of a line position (Dirac delta function) and a line shape (either Lorentzian, Gaussian or mixed) [19]. Mathematically, the infrared absorbance band \( A(\nu) \) in Fig.2-7 can be expressed as a Lorentzian band [22]:

\[
A(\nu) = A_o \frac{\gamma^2}{\gamma^2 + (\nu - \nu_o)^2}
\]  
(2-16)
where $A_o$ is the maximum absorbance of the band, $\nu_o$ is the wave number for $A_o$, and $\gamma$ the half-width at half-height (HWHH).

2.4.2.1 Fourier Self-Deconvolution (FSD) [22]

The cosine Fourier transform of $A(\nu)$ is given by:

$$I(x) = F\{A(\nu)\} = 0.5 \ A_o \ \gamma \ \cos(2\pi \nu x) \ \exp(-2\pi \gamma x) \quad (2-17)$$

where $x$ is a spatial frequency and has the units of cm. The exponential decay term, $\exp(-2\pi \gamma x)$, in Eqn.2-17 is determined by the $\gamma$ value, that is, the wider the absorbance band, the more rapidly its Fourier transform decays. Therefore, if one could decrease the rate of decay of this exponential term, the width of the bands in the infrared spectrum would be reduced. This can be achieved in practice by multiplying $I(x)$ by an exponentially increasing function to yield a new function $I'(x)$. On performing the reverse Fourier transform on $I'(x)$, i.e., $F^{-1}\{I'(x)\} = A'(\nu)$, one obtains a new absorbance band which is narrower than $A(\nu)$. If the new HWHH in $A'(\nu)$ is $\gamma'$, then one can define a ratio of $\gamma / \gamma' = K$ as the resolution enhancement factor. In practice, because the procedure described above also enhances the noise, the new function $I'(x)$ is often smoothed by multiplication with an apodization function.

2.4.2.2 Derivation by Fourier Transforms

An alternative approach to enhancing the resolution of overlapping infrared bands is based on the generation of n-th order derivative band profiles. This can be carried out on the frequency (wavenumber) domain of the spectrum. For a Lorentzian line [22]:

$$A(\nu) = A_o \ \gamma^2 / \left[ \gamma^2 + (\nu - \nu_o)^2 \right] \quad (2-18)$$
The derivatives can be expressed in Equn.3 and the curves in Fig.2-8 accurately fit these expressions [23].

\[ \frac{dA(v)}{dv} = -2(v - v_o)A^2(v) \]
\[ \frac{dA^2(v)}{dv^2} = -2A^2(v) + 8(v - v_o)^2A^3(v) \]  \hspace{1cm} (2-19)
\[ \frac{dA^3(v)}{dv^3} = 24(v - v_o)A^3(v) - 48(v - v_o)^3A^4(v) \]
\[ \frac{dA^4(v)}{dv^4} = 24A^3(v) - 288(v - v_o)A^4(v) + 384(v - v_o)^4A^5(v) \]

2.4.3 Band Assignments of Amide and Side-chains for polypeptides

Polypeptides and proteins exhibit a total of nine characteristic adsorption bands in the infrared region. These are usually termed the amide A, B, and amide I-VII bands (Tab.2-1). The amide I (1700 - 1600 cm\(^{-1}\)) band has been found to be most useful for the study of the secondary structure of proteins.

IR spectra can [24], in principle, be obtained from samples in the solid state or in solution. IR spectroscopy in aqueous solution has always been very difficult because H\(_2\)O absorbs strongly throughout much of the mid-IR region (4000-400 cm\(^{-1}\)). Particular problems are encountered in the conformationally important amide I region (1700-1600 cm\(^{-1}\)) because of the very strong HOH bending mode which occurs around 1644 cm\(^{-1}\). D\(_2\)O can be used instead of H\(_2\)O, thus no strong solvent bands appear close to the amide I frequency region. If D\(_2\)O is used as a solvent, it must be ensured, if possible, that complete H, D exchange has taken place in the backbone amide group.

Table 2-2 lists assignments for amide I band components, based on the study of 19 proteins with known secondary structures. The relative areas of the components provide a good indication of what percentage of the peptide chain is folded into a given
substructure in a given protein. When more than one band is associated with one type of substructure, the sum of the areas of all such bands must be used for this purpose. The procedure is essentially based on empirical observations but, nevertheless, yields quite acceptable results [26,27].

The correlations between common protein structures and the Amide I frequency, which represents the slightly different assignments for amide I band components, have been developed and the results are shown in Table 2-3. Fewer bands of \( \alpha \)-helices and \( \beta \)-sheet, and a wider band width have been used in this method than in that of Byler and Susi’s.

Frequencies and assignments for some protein side chains were also characterized as shown in Table 2-4.

2.4.4 Application of Second Derivative FTIR Spectroscopy [24]

Fig.2-8 shows a single isolated IR band, and its second derivative. For an ideal Lorentzian band, the width of the second derivative is \( 1/2.7 \) of the width of the original band. The second derivative band center is unchanged in frequency, but the peak is now negative with two small positive side lobes. \( A^H \), the peak intensity of the second derivative band (in intensity units/cm\(^2\)) is proportional to \(-A/2\gamma^2\), where \( A \) is the peak height and \( \gamma \) is HWHH of the original band. The relative intensities of second derivative bands can thus be markedly different from those of the original peaks. In particular, narrow features in the original, even if weak, exhibit sharply increased intensities in the second derivative, relative to the peak intensities of second derivatives of broad bands.
The net effect of taking the second derivative of a broad band comprised of a number of overlapping peaks is to resolve the broad band into many of its original components. Because the derivative bands are accompanied by side-lobes, quantitative measurements of the intensities of individual components are very difficult. Second derivative spectra, nevertheless, provide an exceedingly useful fingerprint of individual proteins and effective qualitative means for following subtle changes in their secondary structures.

Although second derivative FTIR is most commonly used in the studies of protein structures and ligand-protein interactions, the 4th derivative was occasionally reported [28].

2.4.5 Application of Fourier Self-Deconvolution — Quantitative Estimation of Protein Secondary Structure

Another most effective procedure of narrowing infrared bands is Fourier self-deconvolution (FSD). FSD is often termed resolution enhancement. However, this terminology is misleading; resolution is an instrumental parameter that cannot be increased after a spectrum is recorded. Procedures such as FSD should therefore more correctly be referred to as band narrowing procedure [46].

As elucidated in equation 2-17 and Fig.2-7, the width of the band will be reduced (band narrowing) when the rate of decay of the exponential term is decreased by multiplying I(x) by an exponentially increasing function. The net effect is to resolve a broad, composite band into its original components [24]. The experimentally measured amide I band of a protein usually shows a broad spectrum from 1700 -1600 cm\(^{-1}\) which consist of many overlapping component bands such as \(\alpha\)-helices, \(\beta\)-structures, turns
and non-ordered or irregular structures. After the broad amide band is decomposed by Fourier self-deconvolution, the areas of the resolved components can be fairly well estimated by curve fitting with Gaussian components.

The methods currently used for quantitative estimation of protein secondary structure can be classified into two categories [45]: those based on band narrowing and decomposition of the amide I band contour into its underlying components (sometimes referred to as “frequency-based” approaches) and those based on the principle of “pattern recognition”. The former is the most commonly used method [46] which involves curve fitting of the amide I after its decomposition. As an initial step, deconvolution is performed to obtain an estimate of the number of discrete absorptions that make up the complex amide I band profile. Curve fitting is often performed on deconvolved spectra, as the individual components are easier to visualize, which in turn makes a visual check of the fit easier [46]. The integrated area under each band after the final iteration is calculated as a percentage of the total amide I area, and this value taken to be the percentage of the particular secondary structure present in the protein. In practice, the amide I’ (1700-1620 cm⁻¹) band is used for deconvolution and curve fitting to estimate protein secondary structure [24,26]. As the positions of the component bands are known, a synthetic curve can be generated by varying the relative intensities and widths of the bands until the synthetic curve matches the experimentally obtained ones. The area of each component band is then computed and this area related to the percentage of protein structure believed to give rise to each band.

Studies of protein structures and ligand-protein interactions by second derivative and FSD techniques have achieved great successes. Nineteen globular proteins with
known secondary structures were investigated by FTIR, resulting in creation of band assignments for amide I band components representing α-helix, β-structures, turns etc. [24, 26]. Great care should be exercised to avoid “over deconvolution” which will cause artifacts [24, 46].

An understanding of the ligand-protein interactions is fundamental to a precise understanding of protein function. It is therefore important that techniques are developed which can be applied to study such phenomena as enzyme-substrate, receptor-hormone, and protein-ion interactions. A large number of important proteins can be shown to be regulated by ion binding, particularly Ca^{2+} binding. Binding positions and structural changes involved in the binding Ca^{2+} ions to a number of proteins have been investigated using FTIR spectroscopy [19].

2.4.6 Choice of FTIR Spectroscopy for Analysis of Protein Secondary Structure [45, 46]

Is FTIR spectroscopy the right technique to use in the analysis of protein secondary structure? It is sufficient to mention at this point that if precise information concerning the relative positioning of individual functional groups in three-dimensional space is required, FTIR is not the technique of choice and X-ray crystallography or NMR spectroscopy may be considered.

While depending to a large extent on the information sought, the question of whether one should use FTIR spectroscopy should also be significantly influenced by our understanding of the comparative strengths and limitations of the many biophysical measurement methods that exist. NMR spectroscopy, for instance, can only presently be applied to proteins with a molecular weight of less than 20,000 Dalton. In addition,
NMR spectroscopy cannot readily be applied to the study of proteins in a membrane environment due to line-broadening effects associated with motional restriction.

Therefore, it can readily be seen that there are many instances, e.g. the analysis of large proteins, where FTIR spectroscopy may be only one of many methods available for characterization. Another major advantage of FTIR spectroscopy for structural characterization is the lack of dependence on the physical state of the sample. Samples may be readily examined as aqueous or organic solutions, hydrated films, in homogeneous dispersions, or solids, and proteins have been analyzed by FTIR spectroscopy in all of these physical states.

Although FTIR spectroscopy is a right technique to choose for the analysis of protein structure in many cases, some potential pitfalls mainly arising from quantitative measurement of protein secondary structure with band narrowing procedure and curve fitting, should be realized as to avoid artifacts in practice.

First commonly encountered pitfall is subjectivity of FSD to choose the deconvolution parameters. If the deconvolution parameters are chosen such that the rate of increase of the exponential corresponds to a band width greater than the width of the absorptions being studied, the result will be sidelobes at the edges of the absorption bands, which can lead to problems with visualization of weaker neighboring bands. On the other hand, if the chosen band width is too small, the adsorption bands will appear unaltered and no additional information will be gained.

Second pitfall is in curve fitting procedure which contains a number of assumptions that are not necessarily justified. The first assumption is that the number and positions of the IR bands that make up the amide I band, as obtained from
deconvolution or derivation, is an accurate reflection of the real number of components. Of course the "real" number of individual amide I band contours is the number of chromophores (number of amide groups) present in the protein. Obviously, the positions of the absorptions arising from each of these chromophores can never be known, except perhaps for very short peptides. In addition to an estimate of the number of amide absorptions, curve fitting routines require the other assumption, i.e. band shape (Lorentzian, Gaussian, or mixed). For simple molecules, IR absorptions are usually assumed to be Lorentzian in shape. However, this assumption does not necessarily hold for larger, complex molecules such as proteins, and the shape of IR absorption arising from proteins is less than clear.

The third pitfall lies in the assignment of each band. Two possible sources of error are inherent in this step. First, it is assumed that the absorptions are due to amide C=O groups involved in secondary structures. In fact, significant absorption in this region of the spectrum may arise from amino acid side chains, including tyrosine, phenylalanine, glutamine, arginine, and lysine [47, 48]. A second source of error lies in the assignment of particular amide I absorptions to specific secondary structures. This is typified by examination of the spectrum of myoglobin, a protein known to be predominantly α-helical in structure, with little or no β-sheet structure present. The IR spectra of myoglobin exhibit an amide I maximum at 1653 cm⁻¹, which is characteristic of proteins with predominantly helical secondary structures. However, a strong shoulder at 1623 cm⁻¹ may be taken as evidence of the presence of a significant amount of β-sheet secondary structures. In the analysis of a particular protein, there are two sets of protein secondary structure assignments available for option. One (in Table 2-2)
is built on the basis of estimations of the secondary structure of some selected "model" protein [24, 27], and the other (in Table 2-3) is developed by combining three approaches such as empirical [25], force-field [49] and dihedral angles [50]. However, the uniqueness in band assignment of protein secondary structure has not been well established in FTIR spectroscopy.

The difficulties highlighted above clearly point to serious limitations and shortcomings of the measurement with band narrowing and curve fitting as a generally valid method to assess quantitatively the "absolute" content of protein secondary structure. However, methods based on band narrowing and curve fitting, if used cautiously, provide a sensitive diagnostic tool for monitoring, in relative terms, the nature of changes in the conformation of the protein backbone.

In addition to the above caveats, another commonly encountered problem is related to the presence of atmospheric water vapor which gives rise to narrow absorption bands in the region overlapping the amide I mode. While these bands are often very weak (or even "invisible") in the original spectrum, because of their relative sharpness compared to that of the amide bands they become disproportionately amplified upon Fourier deconvolution or derivation. These features may appear in the resolution-enhanced spectrum as artifacts that are indistinguishable from the real components of the protein amide band. Therefore, every effort should be made to eliminate water vapor by careful purging of the spectrometer with dry air or nitrogen. If necessary, residual water vapor absorption may be compensated for by spectral subtraction.

2.5 Immunochemical Assays
2.5.1 Basic Concepts and Definition

2.5.1.1 Antigen and haptens

*Antigens or immunogens* [29] are substances that can elicit an immune response. An antigen's immunogenicity is defined as its ability to combine with antibodies produced as a result of acquired immunity to that specific antigen. Antigens are usually of high molecular weight, greater than 1000 Da. *Hapten* [30] is a substance, generally of low molecular weight, that, when injected by itself, does not induce the formation of antibodies; it can induce the formation of antibodies when it is coupled to a protein, polypeptide or other substance to form an “antigenic site”. Antibodies are then induced to the hapten.

2.5.1.2 Antibody Immunoglobulins and the Immune Response

*Immunity* refers to all the mechanisms involved in the mammalian body to protect itself against non-self environmental agents. The initial contact with a foreign agent (antigen immunization) triggers a chain of events that leads to the synthesis of antibodies specific to that foreign agent.

In chemistry, antibodies are multi-chain glycoproteins with a basic structure of two heavy (H) chains and two light (L) chains, which are called *immunoglobulins* (Igs). The basic structure of Igs is symmetrical, and this symmetry allows the immunoglobulin molecule to bind to two identical antigenic determinants. Details of immunoglobulins have been discussed in Chapter 1.

2.5.1.3 Monoclonal Antibodies

Animals produce several clonotype (*polyclonal*) antibodies against a given antigen such that the recovered serum from an immunized animal contains antibodies of many
different classes and specificities. *Monoclonal antibodies* are uniform, specific, and have constant affinity for a single epitope. They are homogeneous in structure and specificity. Monoclonal immunoglobulins are useful for the identification of a single antigen in a complex mixture.

2.5.1.4 Antigen-Antibody Reactions

*Immunogenicity* is the capacity of an antigen to induce an immune response. *Antigenicity* is the ability of an antigen to bind with antibodies or with cells of the immune system. In immunochemistry, antigenicity is involved in the antigen-antibody interactions and can be studied in vitro. These in vitro interactions are used widely in the detection and identification of either antigen or antibodies. Of importance in understanding antigen-antibody interaction are:

(i) The interaction between an epitope (of the antigen) and an antibody does not involve covalent bonds, so the binding forces between a specific epitope and its antibody molecules are relatively weak. The primary chemical interactions consist of Van der Waals, electrostatic, and hydrophobic forces.

(ii) These weak forces require close proximity between the binding site of the epitope and the specific antibody.

(iii) The energy involved in the antigen-antibody interaction is weak and can be broken by high salt concentration by high or low pH, or by chaotropic ions that interfere with hydrogen bonding in aqueous solution.

(iv) The reaction of antigens and antibodies can result in either precipitation (if the antigen is soluble in aqueous medium) or agglutination (if the antigen is particulate).

2.5.1.5 The Use of Labels
Labels in immunochemistry refer to compounds that are chemically attached to one of the assay reactants for the purpose of determining the concentration of that reactant. They allow the antigen-antibody complex to be detected to either the antigen or the antibody.

2.5.2 Enzyme-linked Immunosorbent assay (ELISA)

Many immunochemical assays based on different labelling technologies, such as Radioimmunoassay, Enzyme-linked Immunosorbent assay, Immunofluorescence-assay and Luminoimmunoassay, are currently available. In this section, only Enzyme-linked Immunosorbent assay (ELISA) is discussed.

Any immunoanalytical assay involves four major steps. First, the preparation and purification of the antigen (analyte) to be detected. Second, the production in laboratory animals of an antiserum containing an antibody to the specific antigen. Third, the antibody is recovered, purified, and tested for potency and specificity (avidity) for the tested antigen. Either the antigen or antibody is chemically labeled, but most commonly an enzyme is coupled to the antibody. Finally, an assay is developed for the antigen by monitoring the activity or concentration of the labeled constituent.

2.5.2.1 Classification of ELISA [31]

This section describes six ELISA protocols for the detection of specific antibodies, soluble antigens, or cell-surface antigens. In all six systems, soluble reactants are removed from solution after specifically binding to solid-phase reactants. Table 2-5 summarizes the different ELISA protocols. In the first four protocols, solid-phase reactants are prepared by adsorbing an antigen or antibody onto plastic microtiter
plates; in the next two protocols, the solid-phase reactants are cell-associated molecules. In all protocols, the solid-phase reagents are incubated with secondary or tertiary reactants covalently coupled to an enzyme. Unbound conjugates are washed out and a chromogenic or fluorescent product is generated. Finally, the product is detected visually or with a microtiter plate reader. The amount of product generated is proportional to the amount of analyte in the test mixture.

2.5.2.2 Antibody-sandwich ELISA to Detect Soluble Antigen

Antibody-sandwich ELISAs may be the most useful of the immunosorbent assays for detecting antigen because they are frequently between 2 and 5 times more sensitive than those in which antigen is directly bound to the solid phase (Fig.2-9). To detect antigen, the walls of microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and a different antigen-specific antibody conjugated to enzyme (i.e. developing reagent) is added. After another incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution.

2.6 Chromatographic Separation

Without question, the most widely used means of performing separation is chromatography, a method that finds application to all branches of science. The Russian botanist Mikhail Tswett applied the name chromatography (Greek: chroma meaning “color”, graphein meaning “to write”) to the technique of separating various
chlorophylls and xanthophylls by passing solutions of these compounds through a column packed with finely divided calcium carbonate [32].

Chromatography encompasses a diverse and important group of methods that permit the scientist to separate closely related components of complex mixtures. In all chromatographic separations the sample is run in a mobile phase, which may be a gas, a liquid, or a supercritical fluid. The mobile phase is then forced through an immiscible stationary phase, which is fixed in place in a column or a solid surface. Those components that are strongly retained by the stationary phase move only slowly with the flow of mobile phase. In contrast, components that are weakly held by the stationary phase travel rapidly [32]. In this section, only liquid chromatography, particularly ion exchange and ion exclusion are discussed.

2.6.1 Matrix Materials

2.6.1.1 Commonly used Matrices [33]

The matrix is the solid substrate of the stationary phase. It is modified to confer specific properties on the support by the chemical attachment of various functional groups. As the same functionality may be available on a wide range of matrices it is important to understand the physicochemical properties of the matrices and consider how these influence the selection of a stationary phase (Table 2-6). Ion exchange and ion exclusion based on polystyrene resin have been used in this study.

2.6.1.2 Synthetic Resin —— Polystyrene [34,35]

Polystyrene resin is constructed by polymerizing the styrene with itself and with divinylbenzene (DVB) as a cross-linking agent into a polymeric molecule, which is
used nearly exclusively as the resin matrix in ion exchanger resins of the strongly acidic and strongly basic types [34]:

On the other hand, matrices for weakly acidic cation exchangers are formed, also with DVB as the crosslinking agent, from acrylic acid or methacrylic acid [34]:

Different structures of the resin can be made by use of different proportions of the crosslinking DVB and a solvent to produce various properties for the resins.

2.6.1.2.1 Gel and Macroporous Resins

The term *gel* here refers to the fact, that, macroscopically, a homogeneous network has been synthesized that is elastic in nature and contains solvent from the manufacturing process. It is obvious from this brief description that the term "gel type resins" refers to polymer matrices that do not contain pores [34], but actually contain micropores [35]. Commercial gel type resins contain nominally between 2 and 12% DVB.

Macroporous resins are types in which a non-solvent is used during production from monomers, so that a porous matrix structure is formed in the polymerization. To
prevent collapse of the structure, a large portion of crosslinking agent needs to be used.

2.6.2 Ion Exchange Chromatograph

An ion exchanger is composed of the matrix and ionogenic group (functional group). Ionogenic groups used in ion exchange are, as mentioned earlier, either positively charged (anion exchange resins), or negatively charged (cation exchange resins). Both types can be further divided into strong and weak ion exchange resins. Strong ionogenic groups remain ionized over a wide pH range normally used in separation. Weak ionogenic groups will not be as effective over as wide a pH range as strong ionogenic groups but they can be fully ionized at the optimal pH. The ionogenic groups used in ion exchange chromatography are listed in Table 2-7 [33].

2.6.2.1 Electrostatic Interaction and Retention of Proteins — Net Charge Concept [55, 56]

The ion exchange process is in principle dependent on the attractive forces of oppositely charged particles as defined by Coulomb's law [56]:

\[ F = \frac{(q_1 q_2)}{d r^2} \]

(2-20)

where \( q_1 \) and \( q_2 \) are point charges of opposite sign, \( r \) is their separation distance and \( d \) is the dielectric constant of the medium. The ion exchange process with proteins is considerably more complex since the protein and support are not point charges. However, the proportionality of the relationship remains the same.

The amphoteric nature of proteins is commonly characterized by isoelectric point (pI). Since proteins can exhibit net positive, neutral or net negative charge, depending on their pI and solution pH, the net charge concept has been used to predict retention
behaviour on ion exchange resins based on the assumptions that proteins will not be retained at their pI, that they will be retained by anion exchangers at pH's above their pI or by cation exchangers below their pI and will show a correlation between net charge and retention time [55].

The steps of the protein separation with the ion-exchange process will be briefly explained using a cation-exchanger as an example in Fig.2-10 [37].

2.6.2.2 Deviation of the Net Charge Concept [55, 56]

Although the net charge concept is a theoretical basis for the retention of proteins on ion exchange resins, it is an oversimplified model. Kopaciewicz et al. [55] have demonstrated that approximately three-fourths of the proteins studied deviated from this concept in which they were retained at their respective pI or failed to bind in proportion to net charge when not at their pI. Thus, the importance of protein pI has been overemphasized. Non-systematic deviations from this concept are not the results of non-ionic interactions but, rather, are due to the nature of the protein molecule itself. Intramolecular charge asymmetry promotes differences in electrical potential on the surface of the protein. These regions of localized charge are present even when the net charge of the molecule is zero (at the pI) and postulated to orient or "steer" the protein with respect to oppositely charged surfaces, such as ion exchange support.

2.6.2.3 Influence of Salt on Ion Exchange Chromatography [56, 57, 58]

The strength of the electrostatic interactions between proteins and ion exchange resins decreases with increasing concentration of neutral salts. In ion exchange chromatography it has now been well established [57, 58] that the logarithm of
retention factors (k) decreases linearly with the logarithm of salt concentration. The retention factor is defined as:

\[ k = \frac{t_r - t_o}{t_o} \]  

where \( t_r \) and \( t_o \) are retention times of a retained solute and of an unretained solute respectively. The relationship between \( k \) and salt concentration is represented by the equation [57]:

\[ \log k = z \log a + \log c \]  

where \( a \) is the ionic strength of the mobile phase, \( c \) is a constant, and \( z \) is the slope of the line.

The values of \( z \) and \( c \) have been shown to vary depending on the nature of the salt used for elution. Both the counter ion and the co-ion affect retention times [56, 57]. It was reported [55] that the protein retention of strong cation and anion exchangers was influenced substantially by both the counter-ion and co-ion of the eluting salt.

2.6.2.4 Chemical Equilibrium of Ion Exchange Reactions [36]

Here, a cation exchanger, \( RX^- \), is used to illustrate the principle. The same general considerations apply to an anion exchanger. Associated with \( RX^- \) is a counterion, \( C^+ \). The relative concentrations of free and bound \( C^+ \) are determined by the dissociation constant, \( K_{dc} \):

\[ RX^- C^+ \rightleftharpoons RX^- + C^+ \]  

\[ K_{dc} = \frac{[RX^-][C^+]}{[RX^- C^+]} \]  

When another positively charged ion, \( S^+ \), is added to the system, it binds to the cation exchanger as shown below, and has a dissociation constant \( K_{ds} \).
\[ RX^- S^+ \rightleftharpoons RX^- + S^+ \]  \hfill (2-25)

\[ K_{ds} = \frac{[RX^-][S^+]}{[RX^-S^+]} \]  \hfill (2-26)

The ions C\(^{+}\) and S\(^{+}\) compete for binding to RX\(^{-}\) as shown below. Therefore, S\(^{+}\) and C\(^{+}\) have a partition constant, K\(_p\), in relation to the cation exchanger.

\[ RX^- C^+ + S^+ \rightleftharpoons RX^- S^+ + C^+ \]  \hfill (2-27)

\[ K_p = K_{dc} / K_{ds} \]  \hfill (2-28)

The value of K\(_p\) is a function of experimental conditions.

2.6.3 Ion Exclusion Chromatography [40]

Wheaton and Bauman [38] introduced a separation process in 1953 commonly referred to as ion exclusion which is based on the discovery that certain ion exchange resins, in particular cation exchangers, allow non-ionized solutes such as sugar to penetrate freely into the interior of the resin particle while rejecting electrolytes having the same charge as the functional groups of the exchange resins. The feature of ion exclusion is a separation without the use of chemical regenerants, which is dependent upon the physical and chemical properties of the resin, and no net exchange takes place [39].

The phenomenon of ion exclusion is explained by the Donnan membrane theory [51, 52] in which an unequal distribution of a diffusible electrolyte occurs between two aqueous phases separated by a membrane permeable to water and to both ions of the electrolyte. For such an imbalance to exist there must be present, in addition to a diffusible electrolyte, a large nondiffusible ion on only one side of the membrane. Such a description may be applied to an ion-exchange bead immersed in an aqueous solution
containing an electrolyte. The resin matrix containing the fixed ionic charge constitutes the restricted nondiffusible ion. The boundary surface between the gel-like structure of the ion-exchange bead and the surrounding solution acts as the membrane; water, the counter-ion of the exchanger, and both ions of the electrolyte are permeable to this membrane. When we place the cation exchanger RSO₂⁻Na⁺ in a dilute solution of NaCl and then allow sufficient time for equilibrium to be reached, the concentration of NaCl inside the exchanger is much smaller than that outside. The electrolyte is thus excluded by the resin. The Donnan principle does not apply to uncharged solutes, hence if small enough and not attracted or repulsed by the matrix (e.g. through the influence of van der Waals forces or polar interactions), non-electrolytes will diffuse freely in and out of an ion exchange bead and will tend to have the same concentration both in the resin phase and in the external solution [51].

Thus in these cases, if a sample (such as molasses [40]) containing a non-ionic (e.g. sugar) and ionic (e.g. salt) solute is percolated through a column containing an ion exclusion resin and then the column is rinsed with water, the ionic solute will emerge from the column first, since, due to the Donnan principle, the electrolyte flows around the resin particle but not into it and will emerge when the interstitial volume (the liquid space in between the beads) of the column has been displaced. However, since the nonionic solute diffuses in and out of the resin matrix, it will take a longer time to travel the length of the column and will lag behind the ionic component and emerge from the column last. The ion exclusion resin is usually referred to as the counter-ion carried ion exchanger, and the counter-ion in an exchange resin is sometimes termed as salt form. An illustration of an ion exclusion process used for
separating sugar and ions is shown in Fig.2-11, where separation of non-ionized solutes with electrolytes is based on non-ionized (sugar) travelling along a tortuous way due to penetration into the interior of the resin while the electrolytes keep running "straight".

The cationic counterions of an ion exchange resin, particularly calcium form, may complex with neutral polyhydroxy compounds. It is thought [44] that binding arises from the attraction that the metal (acceptor) ion has for one or more lone pairs of electrons on the donor groups of a ligand (polyols). In complexes where ligands are uncharged, attraction is between the cation and the dipole (static plus induced) of the donor group. Such a ligand-bond is very weak, and water as a hydroxyl compound can replace sugar on Ca\(^{2+}\) form of resin. The most popular application of this principle is for separation of glucose-fructose based on their different strength of binding to the calcium form of the resin (Fig.2-12).

2.6.4 Operative Systems of Chromatography

In general, there are two types of chromatographic operations, namely column and stirred tank systems. The stirred tank system can only be used in "selective adsorption processes" such as ion exchanger and affinity chromatographies. By contrast, the column system can be used in "any process" including those based on partition, e.g. ion exclusion.

In column system, there are two kinds of chromatographic operation [41], namely *frontal chromatography* and *zonal chromatography*.

2.6.4.1 Frontal Chromatography [42]
In frontal chromatography as shown in Fig.2-13 (upper), the feed is continually loaded onto the upper part of adsorbent bed. Saturation of a chromatography column is indicated by the breakthrough point where feed concentration in the effluent continues to rise until the outlet concentration (C) reaches the value of the inlet concentration (C₀). A plot of C/C₀ versus the volume of effluent gives a typical breakthrough curve (Fig.2-13, lower). In most cases a symmetrical curve results and the theoretical capacity of the resin bed is obtained from the volume b, which corresponds to the point C/C₀ = 0.5.

2.6.4.2 Zonal Chromatography [42]

In zonal chromatography as shown in Fig.2-14, the feed is loaded as a small volumes, or as a pulse, onto the top of a packed chromatography column, followed by the eluant. During the elution, those compounds with lower affinities for the adsorbent move faster down the column than those with relatively higher affinities which are retarded. Eventually the components of the feed are resolved into single independent zones. The effluent volume, vₐ or vₐ in Fig.2-14, at which the solute concentration reaches its maximum, is an important constant of elution analysis. This quantity is known as the peak elution volume (or ml to peak) and it determines the chromatographic properties of a solute under a given set of conditions; once known for one column, the ml to peak is readily calculated for columns of other dimensions.

2.6.5 Mathematical Analysis of Zonal Chromatography

2.6.5.1 The Rate of Solute Migration —— Distribution Coefficient, Kᵋ [43]

The terminology of the distribution coefficient (Kᵋ) is used in the analysis of the retention of a solute by ion exclusion chromatography. There are many other
terminologies to express the retention of chromatographic performance, among which the \textit{Capacity Factor} is widely used in the analysis of zonal chromatography [32]. In this section, the description of the \textit{distribution coefficient} ($K_d$) will be based on the mathematical expression introduced by Simpson and Wheaton [43] for polystyrene resin.

In ion exclusion, the \textit{distribution coefficient}, is defined as $K_d = C_r/C_i$ (Fig.2-15a). Here $C_r$ is the concentration of the solute in the resin phase, and $C_i$ is the concentration of the solute in the solution outside of the resin phase.

Wheaton and Bauman [38] have shown that each solute has a definite $K_d$ when placed in contact with ion exchange resins and that these $K_d$ values are functions of the kind of resin and the ionic form of the resin. Electrolytes are excluded from the interior of the resin bead and nonelectrolyte are not, thus there is a difference in the distribution of the electrolyte and the nonelectrolyte between the resin and solution phases.

Generally speaking, a resin bed of total bulk resin volume ($V_T$) can be said to consist of three parts (Fig.2-15a): the void volume (or the interstitial volume) between the resin beads ($V_0$), the occluded volume or the volume of liquid held within the beads ($V_o$), and the volume of the resin network or the solid volume ($V_R$). By applying a mass balance, Simpson and Wheaton [43] showed the equation (Fig.2-15b):

$$C_iV_0 + C_rV_r = C_iV_m$$ \hfill (2-29)

where $V_m$ is effluent volume to maximum concentration.

Dividing by $C_i$ and substituting $K_d$ for $C_r/C_i$, Equation of $K_d$ is finally written as follow:
\[ V_m = V_o + K_d V_r \]  \hspace{1cm} (2-30) \\
\[ K_d = \frac{V_m - V_o}{V_r} \]  \hspace{1cm} (2-31) 

If concentration is expressed as molarity, the capacity factor, \( k' \) can be defined as Moles of A in stationary phase/ Moles of A in mobile phase [53].

\[ k' = \frac{(V_m - V_o)}{V_0} \]  \hspace{1cm} (2-32) 

\( k' \) is obviously a simple and experimentally more accessible term than \( K_d \). Although \( k' \) is a less fundamental factor than \( K_d \) it is nevertheless a commonly accepted way of describing the behaviour of eluates in chromatography [53].

Both \( K_d \) and \( k' \) are based on assuming no forward mixing and complete equilibrium. Even though these conditions are probably not attained, the equation is still a good approximation.

2.6.5.2 Differential Migration Rates — The Selective Factor [32]

The selective factor \( \alpha \) of zonal chromatography for the two species A and B (Fig.2-15b) is defined as

\[ \alpha = \frac{K_d^B}{K_d^A} \]  \hspace{1cm} (2-33) 

where \( K_d^B \) is the distribution coefficient for the more strongly retained species B and \( K_d^A \) is the distribution coefficient for the less strongly held, or more rapidly eluted species A. Thus, Equ.(2-33) can be written as

\[ \alpha = \frac{k'^B}{k'^A} \]  \hspace{1cm} (2-34) 

or

\[ \alpha = \frac{V_m^B - V_o}{V_m^A - V_0} \]  \hspace{1cm} (2-35) 

2.6.5.3 Resolution of Zonal Chromatography [32]
The resolution $R$ of a column provides another quantitative measure of its ability to separate two analytes. $R$ can be defined as (Fig. 2-17)

$$ R = 2 \Delta Z / (W_A + W_B) $$

(2-36)

In practice, the calculation of $R$ will be made by using the distribution coefficient and the selective factor. The equation can be written as

$$ R = N^{1/2} / 4 \cdot (\alpha - 1 / \alpha) \cdot (K_d^B / 1 + K_d^B) $$

(2-37)

2.6.5.4 Quantitative Measure of Chromatographic Column Efficiency — The Plate Theory [32, 42, 43]

The plate theory as applied to chromatography is derived from the mathematical treatment given to the successive equilibriations in a fractional distillation column. The same concept of measuring the efficiency of a distillation column by finding the number of theoretical plates in the column can also be applied to measuring the resolving power of a chromatographic column. A plate is a theoretical transverse layer of cross-sectional area equal to the area of the column. The plate height ($H$) (sometimes referred to by the term used in distillation, height equivalent of a theoretical plate — HETP) is given by

$$ H = L / N $$

(2-38)

where $L$ is the length of (usually in centimeters) of the column packing; $N$ is number of theoretical plates. The efficiency of chromatographic columns increases as the number of plates ($N$) becomes greater and as the plate height ($H$) becomes smaller.

There are four methods used for experimental evaluation of the number of plates ($N$) as diagrammed in Fig. 2-16.

(a) Area-maximum ordinate:
The method involves determining $C_{\text{max}}$ and the total amount of solute, experimentally, from an elution curve \{Fig.2-16(a)\} and then calculating $N$ by means of the expression

$$N = 2 \left( C_{\text{max}} \cdot V_m / M \right)^2$$ \hspace{1cm} (2-39)

(b) Zone-width:

By this procedure, the half-width $W$ of an elution curve at the ordinate value $C_{\text{max}} / e$ (where $e$ is log base) \{Fig.2-16(b)\} is used to calculate $N$.

$$N = 8 \left( V_m / b \right)^2$$ \hspace{1cm} (2-40)

A similar equation of the zone-width method, which has been widely used in HPLC, can be written as [59, 60]:

$$N = 5.54 \left( t_R / W_{0.5} \right)^2$$ \hspace{1cm} (2-41)

where $t_R$ is retention time (the time it takes after sample injection for the analyte peak to reach the detector); $W_{0.5}$ is the peak width at half height.

(c) Tangent:

The third method of calculating $N$ as seen from Fig.2-16(c) is the tangent method. The distance $W$ where the tangents intersect the abscissa is used for the calculation of $N$ according to

$$N = 16 \left( V_m / W \right)^2$$ \hspace{1cm} (2-42)

(d) The method by Simpson and Wheaton:

This method, as shown in Fig.2-16(d), is same as the zone-width in principle. The equation is slightly changed as

$$N = 2 V_m (V_m - V_0) / W^2$$ \hspace{1cm} (2-43)

2.6.5.5 Band Broadening and Plate Height [53, 54]
If thermodynamics were the only controlling factor in chromatography then separations could be effected on very short columns indeed. To illustrate, consider [53] the separation of two solutes whose k' values are, respectively, 1.1 and 1.15, the column volume is 1 ml, the packing is spherical so that \( V_0 \) is approximately 0.35 ml, and the injected volume containing A and B is 0.01 ml. If the separation of the solute band took place without any broadening then a detector sensitive to A and B placed at the column outlet would give two sharp square wave pluses of A and B (Fig.2-18) each 0.01 ml in width, their centers of mass 0.0175 ml apart as calculated from equation 2-29. The two solutes differing only 5% in their distribution coefficients are completely separated — in fact the system is overdesigned in that complete disengagement could be accomplished in a somewhat shorter column in this hypothetical system.

Real systems don't behave in this way with all molecules displaying identical elution times; they display instead a certain distribution of elution times about the mean so that the reality of chromatography is band broadening and more correctly represented by Gaussian-shaped peaks (Fig.2-18).

The Gaussian shape [53] is closely approached if the analyte has undergone a sufficiently large number of sorptions and desorptions. A peak actually represents the frequency distribution of all the molecules of a particular analyte as they move as a group through the system or as they are detected as they exit from the system. The "average" molecule is found at the center of the distribution, at the position of the peak maximum, and it is this "average" position that is used to characterize the particular analyte.

The Gaussian expression linking concentration and volume of the emerging peak is
\[
C = \left\{ \frac{1}{\sigma(2\pi)^{1/2}} \right\} \exp\left[-0.5(V-V_m)/\sigma^2\right]
\]  \hspace{1cm} (2-44)

where \( C \) is the dependent variable, \( V \) is the independent variable, \( V_m \) is the average of a large number of \( V \)'s (effluent volume to maximum concentration), and \( \sigma \) is the standard deviation. In the analysis of peaks resulting from a separation, it will be most useful to express the variable \( V \) in units of standard deviation. Hence, equation (2-44) can be written as:

\[
C = \left\{ \frac{1}{(2\pi)^{1/2}} \right\} \exp(-\sigma^2/2)] = 0.3989 \exp(-\sigma^2/2)
\]  \hspace{1cm} (2-45)

This equation is plotted as a typical Gaussian profile (or distribution) as shown in Fig.2-19. So, \( \sigma \) quantitatively represents band broadening, and it is also in terms of the peak dispersion. As the relationship between \( H \) and \( \sigma \) can expressed as follows [54]:

\[
H = \frac{\sigma^2}{L}
\]  \hspace{1cm} (2-46)

\( H \) is truly the dispersion of an analyte per unit length (column or resin bed) migrated, and \( H \) is the other quantitative measurement of band broadening. A more appropriate term of \( H \) might be column dispersivity.

2.6.5.6 Asymmetry of Gaussian Distribution [54]

Since the Gaussian profile is the ideal shape, an asymmetrical peak is often obtained. Several measures of asymmetry, such as the one shown in Fig.2-20, have been devised by chromatographers for asymmetric peaks. One is called the asymmetric ratio or tailing factor (TF);

\[
TF = \frac{b}{a}
\]  \hspace{1cm} (2-47)

where \( a \) and \( b \) are measured at 10\% of the height of the peak as shown. A symmetrical peak will have a value of 1 and tailed peaks a value of greater than 1. A fronted peak (one with a leading edge and \( a > b \)) will have a value less than 1.
2.7 References


APPENDIX 2-1 TABLES
IN CHAPTER 2
Table 2-1 Characteristic Infrared Bands of the Polypeptide [25]

<table>
<thead>
<tr>
<th>Designation</th>
<th>Approximate&lt;br&gt;Frequency (cm(^{-1}))</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>~ 3300</td>
<td>NH Stretching in resonance with (2x ~ amide II) overtone</td>
</tr>
<tr>
<td>B</td>
<td>3100</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1600 - 1690</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>II</td>
<td>1480 - 1575</td>
<td>CN stretching, NH Bending</td>
</tr>
<tr>
<td>III</td>
<td>1220 - 1301</td>
<td>CN stretching, NH Bending</td>
</tr>
<tr>
<td>IV</td>
<td>625 - 767</td>
<td>OCN bending, mixed with other modes</td>
</tr>
<tr>
<td>V</td>
<td>640 - 800</td>
<td>Out-of-plane NH bending</td>
</tr>
<tr>
<td>VI</td>
<td>537 - 606</td>
<td>Out-of-plane C=O bending</td>
</tr>
<tr>
<td>VII</td>
<td>~ 200</td>
<td>Skeletal torsion</td>
</tr>
</tbody>
</table>

a: based on model compounds
Table 2-2. Characteristic IR frequencies and Assignments for Amide I Band components (1700-1620 cm⁻¹) for 19 globular proteins in D₂O Solution [24]

<table>
<thead>
<tr>
<th>Mean frequency (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1623 +/- 3</td>
<td>β-Structure</td>
</tr>
<tr>
<td>1630 +/- 4</td>
<td>β-Structure</td>
</tr>
<tr>
<td>1637 +/- 3</td>
<td>β Structure</td>
</tr>
<tr>
<td>1645 +/- 4</td>
<td>Unordered (aperiodic)</td>
</tr>
<tr>
<td>1653 +/- 4</td>
<td>α-Helix</td>
</tr>
<tr>
<td>1663 +/- 4</td>
<td>Turns</td>
</tr>
<tr>
<td>1670 +/- 2</td>
<td>Turns</td>
</tr>
<tr>
<td>1675 +/- 5</td>
<td>β-Structure</td>
</tr>
<tr>
<td>1683 +/- 2</td>
<td>Turns</td>
</tr>
<tr>
<td>1689 +/- 2</td>
<td>Turns</td>
</tr>
<tr>
<td>1694 +/- 2</td>
<td>Turns</td>
</tr>
</tbody>
</table>

Table 2-3 Correlations between Common Protein Structures and Amide I Frequency [46]

<table>
<thead>
<tr>
<th>Structure</th>
<th>Amide I frequency (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiparallel β-sheet/aggregated strands</td>
<td>1675 —— 1695</td>
</tr>
<tr>
<td>3₁₀-Helix</td>
<td>1660 —— 1670</td>
</tr>
<tr>
<td>α-Helix</td>
<td>1648 —— 1660</td>
</tr>
<tr>
<td>Unordered</td>
<td>1640 —— 1648</td>
</tr>
<tr>
<td>β-Sheet</td>
<td>1625 —— 1640</td>
</tr>
<tr>
<td>Aggregated strands</td>
<td>1610 —— 1628</td>
</tr>
</tbody>
</table>
Table 2-4  Characteristic Vibrations of the Protein Side Chains [20]

<table>
<thead>
<tr>
<th>Grouping</th>
<th>Vibration</th>
<th>Wavenumber(cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylic</td>
<td>νOH(free)</td>
<td>3560-3500(w)</td>
</tr>
<tr>
<td>Acid (COOH)</td>
<td>νOH(bonded)</td>
<td>2700-2500(w)</td>
</tr>
<tr>
<td></td>
<td>νC=O</td>
<td>1725-1700(s)</td>
</tr>
<tr>
<td>Carboxylate</td>
<td>ν_{as}</td>
<td>1610-1550(s)</td>
</tr>
<tr>
<td>Anion (COO⁻)</td>
<td>ν_{s}</td>
<td>1420-1330(w)</td>
</tr>
<tr>
<td>Phenol</td>
<td>νOH(free)</td>
<td>3612-3592(m)</td>
</tr>
<tr>
<td></td>
<td>νOH(bonded)</td>
<td>3500-3200(s)</td>
</tr>
<tr>
<td></td>
<td>νC-O</td>
<td>1260-1000(s)</td>
</tr>
<tr>
<td>NH₂</td>
<td>ν_{as}NH₂(free)</td>
<td>1669-1610(w)</td>
</tr>
<tr>
<td></td>
<td>ν_{s}NH₂(free)</td>
<td>1550-1485(m)</td>
</tr>
<tr>
<td></td>
<td>νNH₂</td>
<td>3100-2600(s)</td>
</tr>
<tr>
<td>CH₂OH</td>
<td>νOH(free)</td>
<td>3643-3630(m)</td>
</tr>
<tr>
<td></td>
<td>νOH(bonded)</td>
<td>3500-3200</td>
</tr>
<tr>
<td>CH₃OH</td>
<td>νOH(free)</td>
<td>3635-3620(m)</td>
</tr>
<tr>
<td></td>
<td>νOH(bonded)</td>
<td>3500-3200(s)</td>
</tr>
<tr>
<td>ELISA protocol</td>
<td>Uses</td>
<td>Required reagents</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Indirect</td>
<td>Antigen screening; epitope mapping</td>
<td>Antigen, pure or semipure; test solution containing antibody; enzyme conjugate that binds Ig of immunized species</td>
</tr>
<tr>
<td>Direct</td>
<td>Antigen screening; detect soluble antigen</td>
<td>Antigen, pure or semipure; test solution containing antigen; enzyme-antibody conjugate specific for antigen</td>
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<tr>
<td>Antibody-</td>
<td>Antigen screening; detect soluble antigen</td>
<td>Capture antibody (purified or semi-purified specific antibody); test solution containing antigen; enzyme-antibody conjugate specific for antigen</td>
</tr>
<tr>
<td>sandwich</td>
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<td></td>
</tr>
<tr>
<td>Double</td>
<td>Antibody-screening; epitope mapping</td>
<td>Capture antibody; (specific for Ig of immunized species); test solution containing antigen; enzymes-antibody conjugate specific for antigen</td>
</tr>
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<td></td>
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<tr>
<td>sandwich</td>
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<td></td>
</tr>
<tr>
<td>Direct</td>
<td>Screen cells for expression of antigen; measure cellular antigen expression</td>
<td>Cells that express antigen of interest; enzyme-antibody conjugate specific for cellular antigen</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Indirect</td>
<td>Screen for antibodies against cellular antigens</td>
<td>cells used for immunizing; test solution containing antibodies; enzyme conjugate that binds Ig of immunized species</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Matrix</td>
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<td>pH stability</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Whatman Pharmacia-LKB</td>
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</tr>
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<tr>
<td>Polyacrylamide</td>
<td>IBF Bio-Rad Pharmacia-LKB</td>
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<tr>
<td>Silica</td>
<td>Whatman Waters DuPont Merck</td>
<td>3-8</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>Rohm &amp; Haas Dowex Bio-Rad</td>
<td>Any pH</td>
</tr>
</tbody>
</table>
Table 2-7 Ion exchange groups used in the purification of protein [33]

<table>
<thead>
<tr>
<th>Formula</th>
<th>Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strong anion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>— CH$_3$N$^+$(CH$_3$)$_3$</td>
<td>Triethylaminoethyl</td>
<td>TAM</td>
</tr>
<tr>
<td>— C$_2$H$_4$N$^+$(C$_2$H$_5$)$_3$</td>
<td>Triethylaminoethyl</td>
<td>TEAM</td>
</tr>
<tr>
<td>— C$_2$H$_4$N$^+$(C$_2$H$_5$)$_2$CH$_2$CH(OH)CH$_3$</td>
<td>Diethyl-2-hydroxypropylaminoethyl</td>
<td>QAE</td>
</tr>
<tr>
<td><strong>Weak anion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>— C$_2$H$_4$N$^+$(H)$_3$</td>
<td>Aminoethyl</td>
<td>AE</td>
</tr>
<tr>
<td>— C$_2$H$_4$NH(C$_2$H$_5$)$_2$</td>
<td>Diethylaminoethyl</td>
<td>DEAE</td>
</tr>
<tr>
<td><strong>Strong Cation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>— SO$_3^-$</td>
<td>Sulpho</td>
<td>S</td>
</tr>
<tr>
<td>— CH$_2$ SO$_3^-$</td>
<td>Sulphomethyl</td>
<td>SM</td>
</tr>
<tr>
<td>— C$_3$H$_6$SO$_3^-$</td>
<td>Sulphopropyl</td>
<td>SP</td>
</tr>
<tr>
<td><strong>Weak cation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>— COO$^-$</td>
<td>Carboxyl</td>
<td>C</td>
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APPENDIX 2-2 FIGURES
IN CHAPTER 2
Fig. 2-1 Sub-unit model of the casein micelle [3]. (a) Sub-micelles are held together in open disordered structure; lines connecting sub-units denote bridges of colloidal calcium phosphate. (In the refined model, the unconnected sub-micellar surface regions are deemed to be rich in κ-casein.) (b) Each sub-micelle (diameter 10-20 nm) consists of a hydrophobic core (A) of α_{1} -, α_{2} - and β -casein, and hydrophilic coat (B) of κ-casein. (In the refined model, only a limited area of the coat is κ-casein; the rest is composed of hydrophilic residues of α_{1} -, α_{2} - and β -casein.)

Fig. 2-2 Various kinds of colloidal aggregation [4]
Fig. 2-3 Affinity precipitation mechanisms; primary effect (left) and secondary effect (right) [9]
Fig. 2-4 Different membrane processes separate different substances according to size (given in nm). ED, electrodialysis; Nanofiltr., nanofiltration; PV, pervaporation; RO, reverse osmosis [15]
Fig. 2-5 Membrane fouling may be caused by the adsorption of proteins or by pore blocking [15]. Concentration polarization may also lead to membrane fouling if the solute concentration near the membrane interface becomes too high, leading to formation of a gel layer.

Fig. 2-6 Optical diagrams [20] of (a) grating IR photospectrometer (traditional); (b) Fourier transform IR photospectrometer, Michelson type.
Fig. 2-7 Illustration of the process of band-narrowing by Fourier self-deconvolution: The cosine Fourier transform of the original infrared band $A(\nu)$ with a half-width at half-height of $\gamma$ yields the function $I(x)$. Multiplication in the Fourier domain of $I(x)$ with an exponential function $\exp(xy/2.5)$ gives $I'(x)$ which, after reverse Fourier transformation, leads to the new infrared band $A'(\nu)$ whose half-width at half-height is now $\gamma/2.5$.

Fig. 2-8 Comparison of the first, second, third, and fourth derivatives of a Lorentzian line, $E(\gamma)$ [23].
Fig. 2-9 Antibody-sandwich ELISA to detect antigen [31]. Ab = antibody; E = enzyme. Ag = antigen
Fig. 2-10 Schematic diagram for adsorption, elution, fractionation and regeneration of a cation-exchanger [37].
Fig. 2-11 Mechanism of ion exclusion system for separating non-ionic (sugars) and ionic materials. Non-ionic materials (sugars) are penetrated to the interior of the resin bead while ionic materials remain in the exterior of the resin. Ionic matters are eluted ahead of non-ionic matters. Here sugars means glucose, fructose, lactose and sucrose so on.

Fig. 2-12 Mechanism of ion exclusion system for separation of glucose-fructose mixtures. Ligand binding between fructose and Ca^{2+} is stronger than that between glucose and Ca^{2+}. Thus, the glucose is eluted ahead of the fructose.
Fig. 2-13 Schematic representation [41, 42] of frontal chromatography (upper) and a breakthrough curve (lower). The theoretical capacity of the exchanger bed is obtained from volume b, which corresponds to the point \( \frac{C}{C_0} = 0.5 \). The breakthrough capacity of the column is represented by volume a.
Fig. 2-14 Schematic representation [42] of zonal chromatography (upper) and Gaussian-shaped elution curves (lower). Gaussian-shaped elution curves illustrate the separation of two solutes, A and B, by elution analysis. $v_A$ and $v_B$ represent the volume of eluate at which the solute concentrations, respectively, reach their maximum.
Fig. 2-15 Schematic representation of determining distribution coefficient ($K_d$) [43]. (a) represents a magnified section of a column. A resin bed of total bulk resin volume ($V_T$) can be said to consist of three parts: the void volume (or the interstitial volume) between the resin bead ($V_0$), the occluded volume or the volume of liquid held within the beads ($V_R$), and the volume of the resin network or the solid volume ($V_R$). (b) $K_d$ is determined by the position of the elution curve of a solute with respect to the effluent volume.
Fig. 2-16 Schematic representation of determining the number of theoretical plates (N) [42,43]. (a) area-maximum ordinate, (b) zone-width: (c) tangent, (d) the method of Simpson and Wheaton.
Fig. 2-17 Schematic representation of determining resolution (R) [32].

Fig. 2-18 Comparison of "ideal" and real chromatograms [53]. Ideal (no kinetic effects) is represented by the rectangular peaks. Real is represented by the Gaussian peaks (Note: rectangular peaks and Gaussian peaks are on different scales).
Fig. 2-19 A Gaussian shaped peak and determination of the standard deviation $\sigma$ [53].

Fig. 2-20 Figure used to define asymmetric ratio or tailing factor [54].
Chapter 3 Pretreatment of Cheese Whey and Isolation of Lactoferrin by Precipitation of Calcium Phosphate

ABSTRACT

The pretreatment developed in this chapter has simultaneously provided two results for both Cottage and Cheddar cheese whey: whey clarification and isolation of lactoferrin.

After pretreatment, the optical transmission of whey increases to 80-90\% from about 4-40\%, and the flux rate of treated whey on UF membranes can be expected to be dramatically improved. The precipitates account for about 0.6 to 2.0\% of the dry matter in the whey and about 7.3 to 12.3\% of the total protein in the whey.

The pretreatment does not seriously disturb the major whey proteins, α-lactalbumin (La) and β-lactoglobulin (Lg). Only about 5\% of La has been lost in treated whey, and losses of Lg are much less. The studies also indicate that the pretreatment can be operated at room temperature and it doesn’t reduce the content of lactose in supernatant.

Lactoferrin (Lf) has been proved by different analytical methods to coprecipitate with calcium phosphate colloids. The recoveries of lactoferrin by the precipitation step are 66-73\% for cottage cheese whey and 48-72\% for Cheddar cheese whey. The purification factors for lactoferrin based on total protein are measured as 1.8 and 2.9 fold for cottage and Cheddar cheese whey, respectively. Having reduced the mass of the operating material 50-159 times, this precipitation actually is a pre-concentration step for lactoferrin. Lactoferrin in calcium phosphate precipitates can be extracted by 0.5 M NaH₂PO₄ buffer (or 0.95\% NaCl solution) at pH 6-7, where about 80-90\% Lf may be eluted from the precipitate. Lactoferrin may be further purified by CM-chromatography. This process might produce lactoferrin less expensively.

In vitro experimental results have given direct evidence that lactoferrin can coprecipitate with calcium phosphate. A model of pseudo-affinity precipitation involving the formation of a complex of Lf-Ca-PO₄ is proposed to explain the coprecipitation of lactoferrin with calcium phosphate.
3.1 INTRODUCTION

Milk is a colloidal system in which three sorts of colloidal particles, i.e. fat globules, casein micelles, and lipoprotein, are dispersed (Dickinson & Stainsby, 1982). Whey, a by-product of cheese making in which the casein precipitates or fat is removed in some cases, remains a colloidal solution that appears cloudy. The colloidal dispersion in whey is relatively stable; it is observed that there is little sedimentation of aggregates or particles at low centrifugal forces (e.g. 4000 x g) for a certain period of time (i.e. 30 min). Nevertheless, if whey is processed, by ultrafiltration (UF) or column chromatography, aggregation, due to intensified Brownian motion induced by pressure, shearing stress, concentration as well as adsorption onto filters, will result in fouling of membranes (Marshall, 1982) or clogging and channelling in column systems (Kanekanian & Lewis, 1987).

Many pretreatments have been proposed in the last 15 years in order to minimise these problems. The methods concerned can be grouped as: (a) treatment of whey with acid, calcium-sequestering agents, or compounds to modify specific protein side chains or by increasing the ionic strength (Patocka & Jelen, 1987; Lee & Merson, 1976), (b) prefiltration by cross-flow microfiltration (MF) (Lee & Merson, 1976), (c) pretreatment by precipitation of the colloids in HCl casein (room temperature) and cheddar whey (55°C) in the presence of calcium at pH 7.0 followed by centrifugation or MF clarification (Maubois et al., 1987; Kim et al., 1989; Rinn et al. 1990).

The fundamental basis of processes (a) and (c), as explained by some workers (Muller & Harper, 1979; Hayes et al., 1974; Daufin & Michel, 1992), was that the major whey salt, calcium phosphate is sparingly soluble at neutral or alkaline pH so
that aggregation of whey colloidal was stimulated or prevented by addition of calcium or sequesters, respectively.

Caseins are prone to self-association (in the form of the micelle or colloid) due to their high hydrophobicity and peculiar charge distribution (Kanekanian & Lewis, 1987). On the other hand, those caseins with clustering of the phosphoseryl residues such as α_{s1}-, α_{s2}- and β-caseins, are calcium-sensitive and precipitable by calcium to varying extents. As residual caseins probably represent a major form of the colloids in whey, it has been suggested by a logical train of thought that the precipitated colloid is calcium phosphate-casein residual complex (Kanekanian & Lewis, 1987) or an aggregate of phospholipoproteins induced by heat-treatment in the presence of calcium (Maubois et al., 1987). Actually, the constituents of the precipitate formed and the behaviour of the aggregated colloid might be more complicated than those mentioned earlier but have not been examined by other workers.

The objective of this study is to investigate the process involved in the clarification of (cloudy) whey and the selective precipitation of lactoferrin. Large-scale isolation of Lf from whey based on the existing chromatography technology would require large investment and the production cost would be high because the volume of whey to be treated in a large cheese plant is of the order of several hundred thousand litres per day while, in contrast, the concentration of Lf is less than 100 milligrams per litre whey. A commercially viable process to isolate lactoferrin from whey should encompass a simple procedure such as selective precipitation to pre-concentrate the Lf before purification. Redistribution of the Lf to the precipitate would dramatically reduce the volume of liquid to be processed for purifying the Lf fraction. Perhaps the
way to achieve this is to include in the clarification process, a simple step to coprecipitate Lf.

This study deals with investigations of (i) the clarification process including variables and effects of the process, and the behaviour of colloidal aggregation, (ii) identification of lactoferrin in the precipitate, (iii) optimal conditions for isolation of lactoferrin by calcium phosphate precipitation, (iv) the proposed mechanism.

3.2 MATERIALS AND METHODS

3.2.1 Whey samples

Cheddar cheese whey made from whole milk by Norco Co.Ltd., Lismore, New South Wales and cottage cheese whey from skim milk by Australian Cooperative Foods, Hexham, New South Wales, were sampled on the day of cheese manufacture without pasteurization and transported to the laboratory at 5°C. Both cottage and Cheddar wheys were centrifuged by a Sorvall RC-5B at 4000 x g or an Alfa Laval cream separator using relatively large volumes for 30 min at 30°C to remove fat and suspended matters. For the bench experiments, whey was centrifuged as above then packed in a plastic bottle with one litre capacity and frozen at -20°C. Frozen whey was thawed at 2-4°C overnight before pretreatment. For pilot scale experiments, whey was frozen and held at -20°C for not more than 10 days then thawed overnight at 2-4°C before centrifugation followed immediately by pre-treatment. The terms, original cottage cheese whey and defatted Cheddar cheese whey used in this study refer to those samples treated by centrifugation mentioned above. HCl casein whey, which was made
from raw skim milk by precipitation of casein by 1 N HCl at pH 4.6, was sometimes used in the preliminary experiments.

3.2.2 Pretreatment Procedure

To 100 ml or more whey samples solid CaCl$_2$ (as a source of Ca$^{2+}$) and/or NaH$_2$PO$_4$·2H$_2$O (as a source of Pi) were added with agitation until the compounds were dissolved (about 1-2 min), and to 50 ml whey samples the aliquots of CaCl$_2$ (2.5 M) and/or NaH$_2$PO$_4$·2H$_2$O (3.2 M) solutions were added with stirring. Additions of Ca$^{2+}$ and/or Pi were measured in the unit, Millimoles per litre whey (mMoles/L). Increase in volume caused by the addition was never greater than 10% of the initial volume of whey. Whey samples with addition of Ca$^{2+}$ and/or Pi were adjusted to pH 6.5 and above by 6 N NaOH, then centrifuged in the Sorvall RC-5B (Du Pont Instruments) or the Alfa Laval cream separator in the case of using relatively large volumes, at varied centrifugal force for varied time. Except where indicated in the text, room temperature (about 25°C) was always used in the experiments of the pretreatment. In some preliminary experiments, whey samples were merely adjusted to pH 6.5 and above by 6 N NaOH, then centrifuged. Precipitates and supernatants were stored at 5°C, frozen or lyophilized for further experiments. The details of the conditions used for specific experiments will be indicated in appropriate Tables and Figures.

3.2.3 Light Transmission and Absorbance Measurements

Both transmission and absorbance were measured by using a Perkin-Elmer Lambda 2 UV/Vis spectrophotometer at 600 nm with PECSS software version 3.2.

Transmission (%) at 600 nm indicated the efficiency of clarification of whey after the removal of the precipitate, whereas absorbance at 600 nm indicated the turbidity of
whey which was used to monitor aggregation in the pretreatment step based on studies of casein micelle aggregation in milk (Dalgleish, 1983; Bringe & Kinsella, 1993).

In a solution that scatters light, turbidity (absorbance at 600 nm) is proportional to the growth of weight-average molecular weight ($M_w$) of aggregating material (Dalgleish, 1983). Measurement of turbidity allows the detection of colloidal aggregations in the whey, which is induced by factors such as adjustments of pH and additions of calcium and phosphate.

Preliminary experiments indicated that the turbidity of whey, produced by the addition of calcium and phosphorus then adjustment of pH, quickly reached an equilibrium within a couple of seconds and was unchanged in about 5-7 min.

In the experiments on the effects of temperature, and additions of calcium and phosphate on colloidal aggregation, the 10 ml whey samples (original cottage and defatted Cheddar cheese whey) were adjusted to the temperatures of 2°C, 25°C and 50°C, respectively, then different amounts of Ca$^{2+}$ and phosphate (Pi) were added to the whey samples by different aliquots of 2.5 M CaCl$_2$ and 3.2 M Na$_2$HPO$_4$·2H$_2$O solutions. Different volumes of the samples due to the addition were compensated with water. The pH then was adjusted to 7.5-7.8 with 6 N NaOH. The mixtures were kept for 5 min at the temperatures of 2°C, 25°C and 50°C, respectively. The turbidities of the samples were assessed by measuring the absorbances at 600 nm. All these steps were done as quickly as possible, and never took longer than 7 min.

In the clarification experiments, the whey samples (original cottage and defatted Cheddar cheese whey) were treated with environmental factors such as different additions of calcium and phosphate, and at different pHs, and different centrifugal
forces, at room temperature (25°C). The supernatants of treated samples were obtained by centrifugation, and the transmission measured at 600 nm.

3.2.4 Chemical Analysis and Reagents

Estimates of lactose concentration in the supernatants were obtained by measuring the refractive index with an Abbe refractometer. Conductivity was determined by an Activon model 301 conductivity meter. Wet precipitates were weighed as soon as the supernatant solution had been poured off. The centrifuge tube was placed upside down for 2 min to allow more “free water” to drain before weighing. The dry weight was obtained after heating wet precipitates at 105°C in an oven overnight.

A modification of the micro-Biuret procedure (Sleigh, 1982) was used to determine total protein concentration, 0.25 ml of sample being mixed with 1.75 ml water and 1.00 ml reagent. The optical absorbance of the mixture was measured at 310 nm against a blank of reagent plus water. A standard curve was prepared using bovine serum albumin.

The standard protein reagents used including lactoferrin, immunoglobulin G, and lactoperoxidase from bovine milk were the products of ICN Biochemicals, Inc. Bovine serum albumin (BSA) and trypsinogen were from the Sigma Chemical Company. Other reagents used in this study were of analytical grade.

3.2.5 Iron-Binding Capacity of Samples containing Lactoferrin

The iron-binding capacity was determined by a method modified from Graham & Bates (1976), Foley & Bates (1987) and Chen & Wang (1991). Samples in Table 3-2 and Fig.3-18 were dialysed vs EDTA by using the method in 3.2.7 to make Lf in apo-form. A 1.8 ml aliquot of these samples was mixed with 0.2 ml freshly prepared 0.1 M NaHCO₃ then 100 ml 2 mM Fe(NH₄)₂(SO₄)₂ in 0.01 M HCl was added. After
incubation for 30 min at room temperature the absorbance at 465 nm was read against a sample blank. Iron-binding capacity was determined by the following equation. The absorption coefficient, \( E^{1\%} \), 0.57 was used (Chen & Wang, 1991).

\[
\text{Iron-binding Capacity (\%) = } \frac{\text{Absorbance}_{465\text{nm}}}{E^{1\%} \times (0.57)}
\]  

(3-1)

This method was also used to estimate the recovery of Lf from whey.

3.2.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-polyacrylamide gels, PSDS-130, MSDS-1020 and GSS-1020 (Gradiapore Ltd., Sydney), and gel electrophoresis apparatus (Gradiapore Ltd.), were used. Electrophoresis was performed in the buffer system (lower and upper) recommended by Gradiapore Ltd.: Tris 9.2 mM, Tricine 20 mM, SDS 0.1%, pH 7.5. A 10 µl mixture of 50 µl sample, 50 µl 4% SDS, 50 µl 8M urea, 10 µl 2-mercaptoethanol and 10 µl 0.1% bromophenol blue in 50% glycerine solution, was loaded onto the gel and electrophoresed at a constant 150 volts until the bromophenol blue moved out of the gel. Gels were then agitated for 1 hr. in the fixing solution composed of 50% ethanol, 40% water and 10% glacial acetic acid, then stained for 30 min. in a staining solution containing 0.5% coomassie blue, 0.5% amido schwars 10B in the fixing solution. A 3% acetic acid solution was used for destaining.

The periodic acid-schiff (PAS) method (Andrews, 1981) was used for specific glycoprotein staining of lactoferrin. The fuchsin-sulphite (Schiff's reagent) staining solution for this procedure was prepared as follows (Andrews, 1981): 2 g basic fuchsin was dissolved in 400 ml H₂O with warming and then cooled and filtered. To this was added 10 ml 2N HCl and 4 g K₂S₂O₅, and the solution was held cool and dark overnight in a stoppered bottle. Activated charcoal (1 g) was stirred and filtered, and sufficient 2N HCl (10 ml or more) added until a drop dried on a glass slide did not turn
red. The stoppered solution was stored in a cool dark place and discarded if it turned pink. The PAS staining procedure was performed as follows (Andrews, 1981):

1. Electrophoresed gels were immersed in 12.5% TCA for 30 min.
2. Rinsed in H₂O.
3. Immersed in 1% periodic acid in 3% acetic acid solution for 50 min.
4. Washed thoroughly with water (at least 6 changes over 1-2 hr with stirring) overnight.
5. Transferred into Schiff’s stain in the dark for 50 min.
6. Washed with three changes of 0.5% sodium metabisulphite for 30 min.
7. Washed in frequent changes of water until excess stain is removed.
8. Stored in 5% acetic acid.

   Scanning of the stained gel was achieved by densitometry. A photograph of stained gels was scanned by a Shimadzu Dual-Wavelength TCL Scanner CS-910, and plotted using the integrator, LDC/Milton Roy C1-10. Optical transmission was used at a wavelength of 600 nm for all Coomassie blue stained gels. The important parameters in this analysis include slit width (W) and height (H) of light beam, binary attenuation factor (BAF, values from 0 to 9), and chart speed (CS, 0-125 mm/min). For comparison, the same parameters were always used.

   Quantitative measurement of Lf on both Coomassie blue and PAS staining gels was based on Hillier’s method (Hillier, 1976) in which the amount of protein (pₘ) in a sample was found by multiplying the amount of the corresponding protein in the standard protein mixture (pₛ) by the ratio of the peak heights in 2 gels, so

   \[ pₘ = pₛ \cdot \left( \frac{\text{peak height sample}}{\text{peak height standard}} \right) \]  

   (3-2)
In this study, peak height of the sample band was compared with peak height of standard lactoferrin, from ICN Biochemicals, Inc.

3.2.7 Concentration and Dialysis

The concentration was achieved by Centricon-10 (MW cut-off 10 kDa) and Centricon -30 (MW cut-off 30 kDa) membranes from Amicon. Dialysis was performed by a modified method of Masson & Heremans (1968). To the original wheys, supernatants, or redissolved precipitates, 40 mMoles/L EDTA (e.g.1.5 g disodium-calcium salt of EDTA /100 ml solution) was added and the mixture transferred to Spectrapor membrane tubing with MW cutoff 6000-8000, and then dialysed against 20 volumes of the 0.1 M citrate buffer (renewed once), pH 4.0 at 4°C. After 36 hr the citrate was eliminated by dialysis against 20 volumes of deionized water (twice renewed) for 24 hr at 4°C.

3.2.8 Extraction and Purification of Lactoferrin

One-step (Fig. 3-19) and stepwise (Fig. 3-21) extractions for lyophilized precipitates were carried out by using saline solutions and phosphate buffers at different pH, respectively. Experimental details are stated below Figs. 3-19 and 3-21.

A Carboxymethyl-Productiv™ column was used for purification of lactoferrin in the extract. The column was equilibrated with 0.05 M acetate buffer at pH 3.7, then the sample which was prepared by stepwise extraction of the lyophilized precipitate with NaH₂PO₄ solution at pH 6.8 and adjusted to pH 3.7 with acetic acid were applied, followed by elution with a linear gradient of the equilibration buffer and 0.7 M NaCl using a GM-1 mixer (Pharmacia). Fractions of 5 ml were collected by a LKB 2211 SuperRac, LKB BROMMA, and the absorbance measured at 280 nm. Those fractions having absorption peaks were run in SDS-PAGE to identify Lf.
3.2.9 Protein Sequence

The N-terminus of proteins was sequenced to residue 10 on an Applied Biosystems 470A Protein Sequencer by Deakin Research Ltd. Electrophoretic blotting for protein sequencing was run according to Bio-Rad’s protocol for PVDF (polyvinylidene difluoride) protein sequencing membrane.

Mini-gel slab of SDS-polyacrylamide was made in Mini-PROTEAN II Multi-Casting Chamber. Two gels were cast using a formula containing 30% acrylamide stock 4.9 ml, lower Tris Buffer (4x) 2.7 ml, distilled water 2.9 ml, 20% ammonium persulphate (APS) 180 μl and TEMED 5.7 μl. Reagents used and running conditions were as follows:

30% Acrylamide stock: Acrylamide 300 g, Bisacrylamide 8 g, in 1 litre distilled water.
10 x stock Towbin Buffer: 121 g Tris, 576.5 g Glycine in 4 litres distilled water.
(4x) Lower Tris Buffer: 1.5 M Tris, 0.4% SDS, pH 8.8.
(4x) Upper Tris Buffer: 0.5 M Tris HCl, 0.4% SDS, pH 6.8
20% APS (freshly prepared): 0.2 g of APS in 1 ml distilled water.
Running Buffer: 12 g Tris pH 8.5; 57.6g glycine; 2 g SDS in 2 litres distilled water.
Sample Buffer: 4 ml buffer containing 50 mM sodium acetate pH 6.0, 2.5ml; 2% SDS; 6M urea; 100 mM DTT; 5% Bromophenol Blue.
Transfer Buffer: 200 ml 10 x stock Towbin buffer, 400 ml methanol (99.8%) and 3.4 litres distilled water.
Sample: 50 μl + 10 μl sample buffer

Standard Protein: 100 μl per vial, in final 20% sucrose, 2.5% SDS.
Electrophoresis: 100 V through stacking gel, 150 V through separating gel (45min)
Fixative: Methanol:Acetic acid:H₂O with ratio of 40:10:50 (v/v) for 1 hr.
Stain: Coomassie Brilliant Blue 0.025% in 40% MeOH, 1 hr.

Destaining Solution: 20-25% ethanol.

Protein Blotting (Western Procedure) with Bio-Rad’s PVDF membrane was performed as follows:

1). Protein samples were run on SDS-PAGE

2). Part of the gel was removed for protein staining.

3). The PVDF membrane was cut to correct size of the remaining gel and the transfer apparatus (Trans-Blot cell of Bio-Rad Co.) was set up as follows:

4). All were dampened with transfer buffer for 10 min.

5). A weight was applied to top of apparatus to hold all in place.

6). Run conditions included:

   central voltage 300 V

   current limit was adjusted to 110 mA

   the apparatus was run and checked to see if bands on the gel transferred.

7). Proteins-blotted PVDF membrane was ready for protein sequencing.

3.2.10 Experiments for Recovery of Lactoferrin by Precipitation

Additions of calcium and phosphate to the whey were 37.5 mMoles/L Ca\(^{2+}\) + 9.6 mMoles/L Pi for cottage cheese whey, and 74.9 mMoles/L Ca\(^{2+}\) + 9.6 mMoles/L Pi for Cheddar cheese whey. In the case of both wheys, 6 whey samples with 50 ml each
were added. Since calcium phosphate precipitates can be redissolved in the buffer at pH below 4.0, an assumption necessary for calculations of recovery has been made that all Lf is released from precipitates at pH 3.5. Therefore these pH 3.5 solutions of the precipitate were used as the samples for analysis in Table 3-2.

Two samples were used as the control and to four samples were added amounts of Ca\(^{2+}\) and Pi to give wet precipitates. Two wet precipitates were redissolved by 0.1 M citrate buffer at pH 3.5 and the others were extracted with 0.5 M NaH\(_2\)PO\(_4\), pH 6.8 for 20 min to make the precipitates and extracts samples, respectively. All samples including the controls, precipitates (pH 3.5 solutions), and extracts (pH 6.8 extracts), were dialysed by the method in 3.2.7 to make all Lf in apo-form, then made up to the same volume, 70 ml. The samples were filtered through a 0.45 mm membrane before analysis. The analyses included iron-binding capacity, absorbance at 280 nm and SDS-PAGE. The concentrated samples (40 times) were running SDS-PAGE then quantitated. The sample loads were 2 ml.

3.3 RESULTS AND DISCUSSION

3.3.1 The Clarification Process

In a preliminary experiment of this study, acid whey (cottage cheese or HCl casein whey) after adjustment to pH 6.5 and over, suddenly showed a dramatic increase in turbidity then produced a flocculent precipitate. If the precipitates were removed, the supernatant solution was much less cloudy than before. In subsequent experiments of acid wheys such as cottage cheese and HCl casein whey, samples were
adjusted to pH 7.5 and the precipitates removed by centrifugation. The supernatants treated by the process had a transmission of 80-90% as compared with 30-40% for original wheys; and the recovered precipitates represented 14% of the total dry weight in whey. However for Cheddar cheese whey, the process of raising the pH then centrifuging had almost no impact on transmission and precipitation. When Cheddar whey was first adjusted down to pH 4.5 by HCl then raised to pH 7.5 or above by NaOH, the transmission of the supernatant was increased by 10-20% from the initial 4% and more precipitates were obtained. Lowering pH probably allows calcium and phosphate associated with the casein residues in Cheddar whey dissolved.

This phenomenon might be attributed to the fact that Cheddar cheese whey contains less than half of the calcium of acid wheys (Muller & Harper, 1979). Aggregation of colloidal particles in whey is induced by calcium (and phosphate) and alkaline pH (Hayes et al., 1974). Generally, the amounts of calcium (Ca$^{2+}$) of Cheddar cheese whey are in the range of 36.5-47.0 mg/100 ml compared with that of acid whey of about 92.8-150.0 mg/100 ml (Zall, 1992). On the other hand, the contents of phosphorus (Pi) in both sweet and acid whey are relatively close. Cheddar cheese whey contains about 35-43 mg Pi/100 ml compared with 52.6-58.0 mg Pi/100 ml in acid whey (Zall, 1992). In other words, Ca/Pi molar ratio in Cheddar cheese whey is in the range of 0.81-0.84, whereas Ca/Pi molar ratio in cottage cheese whey is in the range of 1.36-2.00. Thus, when the calcium level of Cheddar cheese whey was increased, colloidal aggregation occurred at pH 6.5 or above (Hayes et al., 1974).

The effects of Ca$^{2+}$ (as CaCl$_2$) and Pi (as NaH$_2$PO$_4$) added to whey on clarification and precipitation have been shown in Fig. 3-1 and 3-2. In both cottage cheese and Cheddar cheese whey, the general tendency is similar in that the
transmission of supernatant increased with increase of added Ca\(^{2+}\) and Pi. When 50.0 mMoles/L Ca\(^{2+}\) was added to cottage cheese whey, Pi had little effect on the increase of transmission (Fig. 3-1). Cottage cheese whey added with 50 mMoles/L Ca\(^{2+}\) and more, with or without Pi, has given a supernatant with the transmission of 92-97% (Fig. 3-1) at which the supernatant is transparent (Fig. 3-3a). By contrast, Cheddar cheese whey has shown that the increase of transmission in the supernatant keeps pace with the increase of Ca\(^{2+}\) and Pi until it levels off (Fig. 3-3). Maximum transmission of the supernatant from Cheddar cheese whey, about 85%, can be obtained by addition of 74.9 mMoles/L Ca\(^{2+}\) (or more) and 9.7 mMoles/L Pi, but the supernatant cannot show a completely transparent solution (Fig. 3-3b) by addition of Ca\(^{2+}\) only, which could indicate that the relationship between the calcium content and the colloidal system in Cheddar cheese whey is more complicated than generally considered (Maubois et al., 1987; Kim et al., 1989; Muller & Harper, 1979; Hayes et al., 1974).

In addition, both cottage cheese and Cheddar cheese whey have given the result that the more ions (Ca\(^{2+}\) and Pi) added, the more precipitates were obtained (Fig. 3-1, 3-2), which indicates that added Ca\(^{2+}\) and Pi have formed calcium phosphate precipitates. It is worth mentioning that for cottage cheese whey there were differences in the precipitation from batch to batch. In some batches, clarified whey with 90% transmission could be obtained by merely adjusting the pH to 7.0 or above, followed by centrifugation. But in others, addition of calcium and/or phosphate was critical to achieve the same effects. Such differences probably resulted from the variation of Ca\(^{2+}\) and/or Pi content in whey or changes in the production of cheese.

Effects of addition of Ca and Pi on the variation of total ionic matter (as conductivity) and lactose (as refractive index) in the supernatant have also been tried
and shown in Fig. 3-4. When no Pi is added (series 1 of Fig. 3-4), total ionic matter in
the supernatant increases with the addition of Ca\(^{2+}\). This result implies that, due to less
Pi in the whey, more of the added Ca\(^{2+}\) will remain in the supernatant. In the case of
Pi added with both 3.2 and 9.7 mMoles/L (series 2 and 3 of Fig. 3-4), each has a point
where addition of Ca\(^{2+}\) has decreased the total ionic matter in the supernatant, which
could indicate a stoichiometric relationship between Ca\(^{2+}\) and Pi to form precipitates in
the whey. Fig 3-4 (series 4,5,6) has also shown the effect of the addition of Ca\(^{2+}\) and
Pi on lactose in the supernatant. The results indicate that lactose (as refractive indexes)
in the supernatant has undergone no change with or without ions added under the
conditions used in this study. Precipitation of lactose can be induced by calcium under
alkaline condition. In this process (well known as the Steffen process), lactose can be
recovered from aqueous solution as CaO (Cerbulis, 1973), Ca(OH)\(_2\) (or with NH\(_4\)Cl)
(Olano et al., 1977a), and CaCl\(_2\) together with NaOH (Olano et al., 1977b). The
principle of the Steffen process (obtained from the study of sucrose) is the formation of
a calcium saccharate precipitate by oxides of the alkaline-earth metals (Hartmann,
1974).

Optimal centrifugal forces used for sedimentation of the aggregates have been
investigated and the results shown in Fig.3-5. In general, the aggregates induced by
calcium phosphate require relatively lower centrifugal force and shorter time as shown
in Fig.3-5 where clarification to 94% transmission (a little less than the maximum
96%) can be easily achieved by centrifugation at 2000 x g for 20 min.

It is well known that whey clarification by this method is based on colloidal
aggregation followed by precipitation. The question arises whether temperature is a
factor influencing the aggregation as heating up to 50\(^\circ\)C was used in the process
introduced by Maubois et al. (1987). Effects of temperature with addition of Ca$^{2+}$ and Pi on colloidal aggregation in whey have been shown in Fig. 3-6 and 3-7. In the case of both cottage and Cheddar cheese whey, the samples with the same amounts of Ca$^{2+}$ and Pi added (groups Ca-0, Ca-25 or Ca-75 in Fig. 3-6, 3-7) but treated at a temperature of 2°C, 25°C, and 50°C (curves a, b, c; curves d, e, f; curves g, h, i in Fig. 3-6, 3-7), give very close turbidity measurements (degree of aggregation). Noticeable differences in turbidity are given only by the samples with different amounts of Ca$^{2+}$ and Pi added regardless of the temperature. These results have suggested that degree of aggregation depends upon addition of Ca$^{2+}$ and Pi not on the temperature. It follows that the aggregation by calcium and phosphate in whey over the initial seven minutes is not a function of temperature in the range of 2°C-50°C.

The effects of pH on colloidal aggregation have also been studied, as shown in Fig. 3-8 and 3-9. When to samples of both cottage and Cheddar cheese whey the same amounts of Ca$^{2+}$ and Pi are added, increasing pH values from 6.5 to 9.5 do not greatly influence aggregation of the colloids. Rather, addition of Ca$^{2+}$ and Pi remains a key factor to control colloidal aggregation in the whey. Among these pH values, pH 7.5 or 8.5 is considered more suitable for aggregation. Very high pH (say over pH 9.0) is not practical due to co-precipitation of lactose with Ca$^{2+}$.

The proteins in whey can be co-precipitated with calcium phosphate. Hayes et al. (1974) reported that when the calcium content of a sample of HCl casein whey was increased to 2.7 mg Ca$^{2+}$/ml at pH 7.0 at room temperature the sediment removed by centrifuging at 2500 x g contained 14% of the original protein. The question that might be raised is, what role does calcium and/or phosphate play in the co-precipitation of the protein in whey? Is calcium and/or phosphate the precipitant of the protein in general,
or does it just form a complex with proteins such as casein that is susceptible to calcium. In Fig. 3-10 and 3-11, the relationship between total protein recovery and addition of Ca$^{2+}$ and Pi is presented. In Cheddar cheese whey (Fig. 3-10), recovery of total protein from whey (distribution of the protein in the precipitate) increases with the increase of Ca$^{2+}$ added until it reaches steady levels where the recovery is not affected by further addition of Ca$^{2+}$. In cottage cheese whey (Fig. 3-11), addition of Ca$^{2+}$ does not significantly influence the recovery of total protein probably because Cottage cheese whey already contains a critical concentration of calcium. The recovery (14-16%) of total protein in Cheddar cheese whey is relatively higher than that (10-11%) in Cottage cheese whey. In addition, the effect of Pi on the recovery of the protein is minor (Figs 3-10, 3-11).

Comparisons of the distribution of La and Lg between original wheys and supernatants are shown in Fig. 3-12. Co-precipitated La has been given as 5.2% for Cottage and 4.3% for Cheddar cheese whey, respectively while Lg concentrations distributed in precipitates are less than 1.5% in both wheys. All these results have indicated that only limited amounts of the major whey proteins co-precipitated with calcium phosphate. This is a preferable outcome for a pretreatment, namely that the clarification by the pretreatment should not appreciably occlude the major whey proteins, La and Lg. This result shows an agreement with the results of Kim et al. (1989) in which total whey protein in WPC made from Maubois’ pretreated whey (Maubois et al., 1987) was only 2% less than that for WPC produced from whey. Table 3-1 gives the results of the pretreatment experiments on a pilot plant scale.

3.3.2 Identification of Lactoferrin in the Precipitate
As mentioned above, the pretreatment by using calcium phosphate co-precipitated about 10-14% of the total protein from whey. The proteins contained in such precipitate were regarded as calcium phosphate-casein residual complex (Kanekanian & Lewis, 1987) or phospholipoproteins (Maubois et al., 1987) in previous studies. Whether other interesting proteins are co-precipitated, is a question that should be asked. In preliminary experiments by only adjusting wheys to pH 7.0 and above, the samples of precipitates did show a strong band about 70-80 kDa MW in SDS-PAGE (Fig. 3-13, 3-14). This band was suspected to be lactoferrin.

Bovine lactoferrin and other members of the transferrin family of proteins have been extensively studied. Characteristics such as a molecular weight of about 80,000, the fact that they are glycoproteins and their binding of iron, are well known (see 1.4.3.5 in Chapter 1). The complete protein sequence of bovine lactoferrin was also determined by Pierce et al. (1991). All this evidence provides the criteria necessary to identify lactoferrin in a protein mixture.

Fig 3-13 shows a photograph of an SDS-PAGE gel of samples of cottage cheese whey and fractions. Lane 5 and 10 are samples of precipitates at pH 7.0 and 7.5, respectively. According to Lf standards (lane 6 and 11), the suspected band of precipitates is located in the right position which is about 80 kDa MW, indicated by MW markers. In the case of Cheddar cheese whey (Fig. 3-14), lanes 1-5 are samples of precipitates at different pH in which suspected bands are also running in the same position as the Lf standard (lane 8). As shown in Fig. 3-13, lactoperoxidase (Lp) standards (lane 4 and 9) seem to run a little quicker than Lf bands in both precipitates and standards. The suspected band of precipitates has been also excluded as a possible BSA in comparison with BSA standards (lanes 7 and 12 of Fig. 3-13, lane 6 of Fig. 3-
14). Lane 7 of Fig. 3-14 is bovine immunoglobulin G (IgG) standard. Because 2-mercaptoethanol was used in the preparation of the sample, IgG has been split up to become the bands of heavy and light chains. In this system, the heavy chain of IgG is moving slower than the bands of lactoferrin by comparison between lane 7 and lanes 1-5, and 8 in Fig. 3-14. These results have provided the first evidence to claim that lactoferrin is coprecipitated with calcium and phosphate. In addition, the profiles of SDS-PAGE for defatted (lane 9 in Fig. 3-14) and whole Cheddar cheese whey (lane 11 in Fig. 3-14) are identical, suggesting that centrifugation does not remove the whey protein. In addition, Fig. 3-13 and 3-14 indicate that similar amounts of La, Lg and Lf are contained in precipitates and further suggest that very small proportions of La and Lg have been transferred to precipitates from whey because the ratio of major whey proteins (La and Lg) to Lf is approx. 60:1 in whey (see Chapter 1).

Lactoferrin is a glycoprotein containing “biantennary” glycans of N-acetyllactosamine type α-1,6-fucosylated on the N-acetylglucosamine residues (Spik & Montrenil, 1988). Two types of primary structures of a biantennary glycan from bovine milk Lf are as follows residues (Spik & Montrenil, 1988):

Gal (a 1-3) Gal (b 1-4) GlcNAc (b 1-2) Man (b 1-6) \[\text{ NeuAc (a 2-6) Gal (b 1-4) GlcNAc (b 1-2) Man (a 1-3)}\]
\[
\text{ NeuAc (a 2-6)}\,_{\text{5-1}} \text{ GalNAc (b 1-4) GlcNAc (b 1-2) Man (b 1-6)}
\]
\[
\text{ NeuAc (a 2-6) Gal (b 1-4) GlcNAc (b 1-2) Man (a 1-3)} \quad \text{ Man (b 1-4) GlcNAc (b 1-4) GlcNAc (b 1-N) Asn (Fuc)}
\]

The glycoproteins can be stained as a pink colour by Schiff’s reagent. Fig. 3-15 gives the result of PAS staining of the precipitates compared with Lf and Lp standards.
Lane 2 is the sample of precipitates from cottage cheese whey and it shows a clear pink band which runs in the same position as that of the Lf standard (lane 3) and gives a similar band shape. On the other hand, Lp (a glycoprotein, as well) standard (lane 1) is obviously moving quicker (smaller MW) than the bands of Lf and has a much less dense band.

Fig. 3-16 displays the iron-binding characteristic of precipitates from cottage cheese whey. The extract of precipitates with added Fe(NH₄)₂(SO₄)₂ in 0.01 M HCl (curve b of left panel) has given a small but clear peak (absorption) at about 450-470 nm which indicates the complex of Iron-Lf-CO₃²⁻. By contrast, the extract without added Fe(NH₄)₂(SO₄)₂ in 0.01 M HCl (curve a of left panel) hasn’t shown that absorption at all. The difference spectrum of curve b versus curve a in the right panel also gives an absorption at about 450-470 nm. This result has strongly suggested that the precipitate has iron-binding activity.

The last effort to prove that lactoferrin coprecipitated with the precipitate of calcium phosphate was to use the powerful method — analysis by protein sequencing. Fig. 3-17 has illustrated the Western procedure of protein blotting from stained gels to stained PVDF membranes from which a single band (the highly purified polypeptide) is cut for the protein sequencer. There are about 9 single polypeptides blotted with light or dense bands in the sample. The N-terminal sequences of the first and last band (from top to bottom) were determined to residue 10 and are given as follows:

First band (about 80 kDa MW): I A P R K N V R W C T 10

Last band (about 14 kDa MW): I E Q L T K C E V F R 10

As compared with known sequences of the proteins, the N-terminal 10 residues of the first band are identical with that of lactoferrin for species *Bos Primigenius*
*Taurus* (Pierce et al., 1991) whereas the N-terminal 10 residues of the last band are identical with that of bovine \( \alpha \)-lactalbumin (Shewale et al., 1984). Therefore, the dense band with 80 kDa MW (in Fig. 3-17) has been confirmed to be lactoferrin.

### 3.3.3 Optimization for Precipitation, Extraction and Purification of Lactoferrin

As Lf has been shown to be coprecipitated with calcium phosphate, studies proceeded on with factors and methods which would lead to the setting up of a process, including precipitation, extraction and purification of lactoferrin from whey.

Fig. 3-18 shows the effects of adding Ca\(^{2+}\) and Pi on the distribution of lactoferrin in the precipitates from cottage cheese whey. The results have indicated that without adding Pi, iron-binding capacities (as Lf contents) in the precipitates remain at a low level and there are no significant increases when Ca\(^{2+}\) is added, but when phosphate is added more lactoferrin has been distributed to the precipitates.

Results for the extraction of Lf by saline solution (0.95% NaCl) at different pH's and precipitate concentrations have been given in Figs. 3-19 and 3-20. At pH 6.0, most Lf, 12 mg Lf /g precipitates, was extracted by saline solution, which is a little higher than those at pH 2.0 and 8.0, 10 mg and 11.67 mg Lf /g precipitates, respectively. However, extraction of Lf at pH 10.0 is very poor and only about a half of the pH 6.0 extract, 6.33 mg Lf /g precipitates, was obtained. On the other hand, the purity of Lf in the extracts is increased with the increase in the pH and a higher purity is observed in a lower precipitates concentration in the solution. These results have suggested that all proteins are extracted with greater difficulty from calcium phosphate at higher pH (e.g pH 10.0), but lactoferrin is more weakly bound to calcium phosphate than others at that pH, resulting in extraction of the Lf in low recovery but high purity. It is easily understood that the Lf extracted at lower pH (e.g. pH 2.0) must have lower purity
because of dissociation of the calcium-phosphate-protein complex at that pH. Based on this reasoning, the recovery of Lf should be highest at acidic pH. However, the result is in conflict. This could be caused by unstabilized casein in acidic condition bringing down some Lf during centrifugation, especially as Lf is known to interact with casein (Oram & Reiter, 1968). In addition, salts like NaCl or KCl can cause the elution of basic proteins (such as lactoferrin), but not of acidic proteins from hydroxylapatite (a form of calcium phosphate) (Bernardi et al. 1972). Fig. 3-20 gives the scanning profiles of SDS-PAGE gels for the experiments in Fig. 3-19.

If the precipitate containing the complex of calcium-phosphate and Lf is treated as an Lf-adsorbed hydroxylapatite resin, Lf should be extracted by phosphate buffer. In general, protein elution from hydroxylapatite is achieved with a stepwise or continuous gradient of increasing phosphate concentration up to 500 mM around pH 6.8 (Roe, 1989). Lactoferrin elution from the precipitate by successive extraction has been carried out using a stepwise gradient of increasing phosphate concentration from 0.01-1.0 M. The results are shown in Figs. 3-21 and 3-22. In the case of pH 6.0 (Fig. 3-21,c), the profile of the extraction suggests that Lf is dissolved from the precipitate at phosphate concentrations more than 0.1 M. But using pH 8.0 (Fig. 3-21,d), the profile of Lf extraction seems to have two stages. Lf is first extracted at 0.01 M phosphate whereas the rest of Lf is removed at phosphate concentrations of more than 0.1 M. If this precipitate is extracted again at pH 8.0 (Fig. 3-21,d), 1.0 M phosphate can no longer extract Lf, thus indicating that all removable Lf has been dissolved. In addition, the patterns for profiles of total protein extraction at both pH 6.0 (Fig. 3-21,a) and 8.0 (Fig. 3-21,b) coincide with those corresponding to Lf extraction. Fig. 3-22 gives the scanning profiles of SDS-PAGE gels. Evidently the extraction in 0.5 M
phosphate at pH 6.0 (Fig. 3-22,d) shows the least impurity. This result has indicated that in order to get relatively pure Lf in the extract, the practical way for eluating Lf is to use repeated extraction or less concentrated precipitate solutions in 0.5 M phosphate buffer, pH 6.0-7.0.

By way of summing up the results, a process to isolate lactoferrin from whey is suggested in Fig. 3-23. The performance of the critical stages in this process including precipitation and extraction, have been tried and the results are given in Table 3-2.

In the case of cottage cheese whey, the content and iron-binding capacity of lactoferrin in original whey were determined as 12.39 and 2.38 mg/100ml, respectively. The yield of Lf and iron-binding capacity produced by calcium phosphate precipitation were measured as 8.19 and 1.74 mg/100ml, separately. The recovery of lactoferrin by precipitation from whey is 66% or 73%, whereas the extraction recovery of lactoferrin calculated on the basis of whey is 62% or 67% according to the scanning method or measurement of iron-binding capacity. Thus, over 90% of lactoferrin can be extracted from the precipitate by 0.5 M NaH₂PO₄ at pH 6.8. The $A_{280} / A_{470}$ value of pure lactoferrin was reported (Foley & Bates, 1987) as about 20. The purification factors of lactoferrin by precipitation and extraction according to $A_{280} / A_{465}$ method are calculated as 1.8 and 2.6 fold.

In the case of Cheddar cheese whey, the content and iron-binding capacity of lactoferrin in original whey were determined as 9.3 and 1.66 mg/100ml, respectively. The yield of Lf and iron-binding capacity produced by calcium phosphate precipitation were measured as 4.46 and 1.2 mg/100ml, separately. The recovery of lactoferrin by precipitation from whey is 48% or 72% whereas the extraction recovery of lactoferrin calculated on the basis of whey is 45% or 57% according to the scanning method or
measurement of iron-binding capacity. Thus, about 80-90% of the lactoferrin can be extracted from the precipitate by 0.5 M NaH₂PO₄ at pH 6.8. The purification factors (folds) of lactoferrin by precipitation and extraction with using $A_{280}/A_{470}$ method are calculated as 2.9 and 3.4 fold.

The comparison of the results for cottage and Cheddar whey has indicated that recoveries of Lf for cottage cheese whey by precipitation and extraction are higher than those for Cheddar cheese whey. This is probably because Cheddar cheese whey contains much more colloid than Cottage cheese whey, and these extra colloids could compete with Lf and prevent it from complexing with calcium phosphate. Furthermore the colloids contained in the precipitate might prevent Lf from being removed by extraction. The extent of purification of Lf from Cheddar cheese whey is higher than that from cottage cheese whey. Because of the higher ratio of total protein to Lf (bigger value of $A_{280}/A_{470}$) in Cheddar cheese whey, this result has implied that the precipitation by calcium and phosphate will be performed more efficiently in a protein mixture containing less of the target protein. In addition, the recovery obtained by the scanning method is much lower than that by measuring iron-binding capacity in Cheddar cheese whey, but the recoveries measured by different methods are relatively close each other in cottage cheese whey. This error in the experiments was caused by higher contents of residual casein and other colloids in Cheddar cheese whey which can also complex Lf and might make Lf deposit on the membrane during concentration, or retard the mobility of Lf in running SDS-PAGE gel.

Very pure lactoferrin can be produced by different chromatographic methods which have been well described (Foley & Bates, 1987; Chen & Wang, 1991; Kawakami et al., 1987; Yoshida, 1989; Law & Reiter, 1977; Yoshida & Yun, 1991;
Dionysius et al. (1991). Among them, carboxymethyl (CM) cation exchange chromatography reported by Law & Reiter (1977), Yoshida & Yun (1991), and Dionysius et al. (1991) is most practical for the purification of Lf from whey. CM-Productiv™ column and an acidic chromatographic condition (pH 3.7) were used to fractionate the extract of the precipitate, as shown in Fig.3-24. The last peak in the profile has been identified by SDS-PAGE as containing Lf, which can be expected because Lf as a slightly basic protein has stronger positive charges at pH 3.7.

3.3.4 “In vitro” study of the precipitation of lactoferrin and proposed mechanism

Although many experiments mentioned above have already suggested that lactoferrin co-precipitates with calcium phosphate, direct evidence for this is shown in Fig. 3-25. When standard Lf solution was added to Ca$^{2+}$ and Pi and then adjusted to pH 7.5, the precipitated fraction has been found to contain protein (according to absorption at 280 nm) and the concentration is increased slightly with the increase in calcium added (Fig. 3-25,A). The fractions of redissolved precipitates and the supernatants were run by SDS-PAGE (Fig. 3-25,B). The supernatants had completely lost lactoferrin, which, however, has been found in the precipitate. This experiment has given clear evidence that lactoferrin can co-precipitate with calcium phosphate.

Based on current knowledge of ligand-protein binding and affinity precipitation, this study attempts to propose the terminology and a model for the precipitation of Lf by calcium phosphate.

In typical affinity precipitation (Chen, 1990) the ligand is attached to a soluble carrier to form a macroligand. Complexes formed between target proteins and water-soluble macroligands can precipitate out of the solution while contaminants are left in the supernatant solution. There are two ways to induce precipitation (Chen, 1990),
depending upon the nature of macroligands, namely homo-bifunctional ligands and hetero-bifunctional ligands (see 2.2 of Chapter 2). An example of metal affinity precipitation was demonstrated with bis-chelates of \( \text{Cu}^{2+} \) as macroligands (Chen, 1990).

It has been reported (Bennett et al. 1981) that a tetramer of lactoferrin can form in the presence of \( \text{Ca}^{2+} \). \( \text{Ca}^{2+} \) has also been found in this thesis to bind to carboxylic side-chains of Lf (see Chapter 4). Such a ligand-binding characteristic of \( \text{Ca}^{2+} \) to Lf agrees with the principle of affinity precipitation. So, coprecipitation of Lf with calcium phosphate might be considered as calcium affinity precipitation rather than as a protein adsorbed onto hydroxyapatite in general. The result in Fig. 3-12 will support this suggestion as a noncalcium-binding protein, \( \beta \)-lactoglobulin, wasn’t significantly lost with the addition of calcium and phosphate. However, in a typical affinity precipitation the ligand is first attached to a carrier to form a macroligand before it is used to entrap target protein. In this study, the procedures such as ligand (\( \text{Ca}^{2+} \)) attaching to a carrier (\( \text{PO}_4 \)), ligand (\( \text{Ca}^{2+} \)) binding with target protein (Lf), and crosslinking to precipitation are carried out simultaneously. From this aspect, the precipitation of lactoferrin with calcium phosphate is more like agglutination. So, \textit{pseudo-affinity precipitation} may be an appropriate terminology for this precipitation of lactoferrin, the mechanism for which has been proposed in Fig. 3-26. In a mixture of Lf, \( \text{Ca}^{2+} \) and phosphate, each component is in a free state at acidic pH (\( \text{Ca}^{2+} \) may weakly bind to Lf). When the mixture is adjusted to pH 7.5 or over, \( \text{Ca}^{2+} \) is binding to the carboxylic groups of Lf and a tetramer of Lf is formed. \( \text{Ca}^{2+} \) in the tetramer is linked with phosphate to form the complex of Lf-\( \text{Ca}^{2+} \cdot \text{PO}_4 \) which further crosslinks to form a three-dimensional network structure and eventually precipitates.
3.4 CONCLUSION

The pretreatment technology developed in this study has simultaneously provided two processes on both Cottage and Cheddar cheese whey, namely whey clarification and isolation of lactoferrin.

The pretreatment process can be described as follows. Whey is centrifuged at 37°C to remove large particles of casein and fat. To cottage cheese whey is added 37.5 mMoles/L Ca\(^{2+}\) and 9.6 mMoles/L Pi, and to Cheddar cheese whey is added 75 mMoles/L Ca\(^{2+}\) and 9.6 mMoles/L Pi with stirring. Whey with Ca\(^{2+}\) and Pi added is adjusted to pH 7.5 with 6 N NaOH. After standing for 10 min, such whey is centrifuged at 3000 x g to give the precipitate and supernatant (treated whey).

After treatment, the optical transmission of whey increases to 80-90% from about 4-40%, and the flux rate of treated whey on UF membranes can be expected to be dramatically improved.

Based on experiments of pilot plant scale, the yields of dry matter in precipitates are about 0.6-2%, and about 7.3-12.3% of total protein in whey has been recovered by precipitation. In addition, the mass reduction of dry precipitates vs. whey is 50-159 times.

As the results in this study have indicated that colloidal aggregation over the initial seven minutes is not the function of the temperature in the range of 2-50°C, the process can be operated at room temperature.

Lactose is not disturbed by calcium phosphate precipitation as its concentration in the treated whey remains unchanged. The pretreatment doesn’t seriously coprecipitate
the major whey proteins, α-lactalbumin and β-lactoglobulin, either. Only about 5% of La has been lost in treated whey, and losses of Lg are very small.

Lactoferrin has been proved by different analytical methods to coprecipitate with calcium phosphate colloids. The recoveries of lactoferrin by the precipitation step are 66-73% for cottage cheese whey and 48-72% for Cheddar cheese whey. The purification factors of lactoferrin based on total protein are measured as 1.8 and 2.9 fold for cottage and Cheddar cheese whey, respectively. Having reduced the mass of the operating material 50-159 times, this technology by precipitation actually is a pre-concentration step for lactoferrin. This effect is particularly important for the industrial angle because a factory is facing a million litres whey containing lactoferrin at about 2-35 mg /100 ml (Table 1-4 in Chapter 1). The pre-concentration provided by one step precipitation can be expected to produce lactoferrin with a cheaper price.

Lactoferrin can be extracted by 0.5 M NaH₂PO₄ buffer (or 0.95% NaCl solution) at pH 6-7, where about 80-90% Lf may be eluted from the precipitate. Lactoferrin can be further purified by CM-chromatography.

"Invitro" experimental results have given direct evidence that lactoferrin can coprecipitate with calcium phosphate. As compared with the principle of typical affinity precipitation, this method for isolating Lf from whey has been considered as pseudo-affinity precipitation. A model is proposed to demonstrate the formation of the complex of Lf-Ca²⁺-PO₄.
3.5 REFERENCES


APPENDIX 3-1: TABLES IN CHAPTER 3
Table 3-1
Pretreatment of the Cheese Wheya

<table>
<thead>
<tr>
<th></th>
<th>Input (kg)</th>
<th>Wet Pptes (kg)</th>
<th>Dry Pptes (g)</th>
<th>Mass reduction (fold)</th>
<th>Transm. (%)</th>
<th>Protein Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCCW^b</td>
<td>420</td>
<td>38.2</td>
<td>8400</td>
<td>11^c 50^d</td>
<td>80</td>
<td>12.3</td>
</tr>
<tr>
<td>OCCW^b</td>
<td>54</td>
<td>1.5</td>
<td>338</td>
<td>36^c 159^d</td>
<td>90</td>
<td>7.3</td>
</tr>
</tbody>
</table>

a: The experimental conditions were: For Cheddar cheese whey, 74.9 millimole Ca^{2+} and 9.6 millimole Pi per liter whey were added. For cottage cheese whey, nothing was added. The wheys were warmed to 20°C and adjusted to pH 7.5. The mixtures were maintained for 15 min before centrifugation to collect the precipitates.
b: DCCW, defatted Cheddar cheese whey, OCCW, original cottage cheese whey.
c: Mass reduction = whey (kg) / wet precipitates (kg).
d: Mass reduction = whey (kg) / dry precipitates (kg).
e: Optical transmission of treated whey.
Table 3-2
Recovery and Purification of Lactoferrin by Precipitation and Extraction

<table>
<thead>
<tr>
<th>Cottage cheese whey</th>
<th>Yield(^a) (mg/100 ml whey)</th>
<th>Capacity(^b) (mg/100 ml whey)</th>
<th>Recovery (%</th>
<th>(A_{280}/A_{470})</th>
<th>Purification Factor (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey</td>
<td>12.49</td>
<td>2.38</td>
<td></td>
<td>754(696-811)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.2-2.55)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pptes(^c)</td>
<td>8.19</td>
<td>1.74</td>
<td>66(^a), 73(^b)</td>
<td>412(264-560)</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.12-2.35)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracts</td>
<td>7.77</td>
<td>1.64</td>
<td>62(^a), 67(^b)</td>
<td>288(281-295)</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.6-1.68)</td>
<td></td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Cheddar cheese whey</th>
<th>Yield(^a) (mg/100 ml whey)</th>
<th>Capacity(^b) (mg/100 ml whey)</th>
<th>Recovery (%</th>
<th>(A_{280}/A_{470})</th>
<th>Purification Factor (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey</td>
<td>9.3</td>
<td>1.66</td>
<td></td>
<td>1362</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.61-1.7)</td>
<td></td>
<td>(1333-1391)</td>
<td></td>
</tr>
<tr>
<td>Pptes(^c)</td>
<td>4.46</td>
<td>1.2</td>
<td>48(^a), 72(^b)</td>
<td>466</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.99-1.41)</td>
<td></td>
<td>(384-548)</td>
<td></td>
</tr>
<tr>
<td>Extracts</td>
<td>4.26</td>
<td>0.95</td>
<td>45(^a), 57(^b)</td>
<td>395</td>
<td>3.4</td>
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<tr>
<td></td>
<td></td>
<td>(0.9-1.0)</td>
<td></td>
<td>(367-423)</td>
<td></td>
</tr>
</tbody>
</table>

The experimental conditions were shown in 3.2.10.
The data are the average of duplicates. Numbers in parentheses give the range.

\(^a\): Yield was obtained by scanning SDS-PAGE gels.

\(^b\): Iron-binding capacity was measured by the Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\) method.

\(^c\): Precipitates that were analysed by using their pH 3.5 solutions.

Recovery (%) = ratio of the yield (or capacity) of the Pptes and extract to the yield (or capacity) of the control whey.

Purification factor = ratio of \(A_{280}/A_{470}\) in the control whey to \(A_{280}/A_{470}\) in the Pptes and extract.
APPENDIX 3-2 FIGURES
IN CHAPTER 3
Fig. 3-1 Effects of Addition of Ca\(^{2+}\) and Pi on Supernatant Transmission and Precipitate Weight in Cottage Cheese Whey. Curves of Series 1, 2 and 3 are relative to wet weight, and curves of Series 4, 5 and 6 to transmission %. Series 1 and 4: [Ca\(^{2+}\)] only; Series 2 and 5: [Ca\(^{2+}\)] + 3.2 millimoles P (phosphorus); Series 3 and 6: [Ca\(^{2+}\)] + 9.7 millimoles P (phosphorus).
To 100 ml samples of original Cottage cheese whey different amounts of Ca\(^{2+}\) and Pi were added. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then centrifuged at 5000 x g for 30 min. Transmission and wet weight were determined on supernatants and precipitates, respectively.

Fig. 3-2 Effects of Addition of Ca\(^{2+}\) and Pi on Supernatant Transmission and Precipitate Weight in Cheddar Cheese Whey. Curves of Series 1, 2 and 3 are relative to wet weight, and curves of Series 4, 5 and 6 to transmission %. Series 1 and 4: [Ca\(^{2+}\)] only; Series 2 and 5: [Ca\(^{2+}\)] + 3.2 millimoles P (phosphorus); Series 3 and 6: [Ca\(^{2+}\)] + 9.7 millimoles P (phosphorus).
To 100 ml samples of defatted Cheddar cheese whey different amounts of Ca\(^{2+}\) and Pi were added. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then centrifuged at 5000 x g for 30 min. Transmission and wet weight were determined on supernatants and precipitates, respectively.
Fig. 3-3 Photograph of whey before and after treatment. (A) left: original cottage cheese whey (ACF), right: pretreated cottage cheese whey. (B) left: defatted Cheddar cheese whey (Norco), right: pretreated Cheddar cheese whey.

Pretreatment was carried out as follows: the aliquots of 37.5 (mMoles/L) Ca\(^{2+}\) + 9.6 (mMoles/L) P\(^*\) and 74.9 (mMoles/L) Ca\(^{2+}\) + 9.6 (mMoles/L) P\(^*\) were added to 300 ml samples of the original cottage and defatted Cheddar cheese whey, respectively. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then centrifuged at 5000 x g at 25°C for 30 min to get the supernatant solution.

*P: Phosphorus.
Fig. 3-4 Effects of Addition of Ca^{2+} and Pi on Total Ionic Matter and Lactose in the Supernatant of Cheddar Cheese Whey. Curves of Series 1, 2 and 3 refer to conductivity, and curves of Series 4, 5 and 6 to refractive index. Series 1 and 4: [Ca^{2+}] only; Series 2 and 5: [Ca^{2+}] + 3.2 millimoles P (phosphorus); Series 3 and 6: [Ca^{2+}] + 9.7 millimoles P (phosphorus).

To 100 ml samples of defatted Cheddar cheese whey different amounts of Ca^{2+} and P were added. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then centrifuged at 5000 x g for 30 min.

Conductivity and refractive index were determined on supernatants.

Fig. 3-5 Optimization of Centrifugal Force for Clarification of Whey. To 100 ml samples of cottage cheese whey 60 (mMoles/L) Ca^{2+} and 3.2 (mMoles/L) P (phosphorus) were added. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then centrifuged by Sorvall RC-5B using different centrifugal forces (x g) and times. Optical transmission on the supernatant was determined at 600 nm.
Fig. 3-6 Effect of Temperature on Colloidal Aggregation in Cottage Cheese Whey. Ca-0 represents curves a, b and c: without addition of Ca$^{2+}$; Ca-25 represents curves d, e and f: addition of 25 mMoles/L Ca$^{2+}$; Ca-75 represents curves g, h and i: addition of 75 mMoles/L Ca$^{2+}$.

To 10 ml samples of the original cottage cheese whey different amounts of Ca$^{2+}$ and Pi were added. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then treated at temperatures of 2°C, 25°C and 50°C for 5 min. Absorbance as turbidity of the samples were determined at 600 nm.

Fig. 3-7 Effect of Temperature on Colloidal Aggregation in Cheddar Cheese Whey. Ca-0 represents curves a, b and c: without addition of Ca$^{2+}$; Ca-25 represents curves d, e and f: addition of 25 mMoles/L Ca$^{2+}$; Ca-75 represents curves g, h and i: addition of 75 mMoles/L Ca$^{2+}$.

To 10 ml samples of the defatted Cheddar cheese whey different amounts of Ca$^{2+}$ and Pi were added. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then treated at temperatures of 2°C, 25°C and 50°C 5 min. Absorbance as turbidity of the samples were determined at 600 nm.
Fig. 3-8 Effect of pH on Colloidal Aggregation in Cottage Cheese Whey. To 100 ml samples (from S-1 to S-9) of the original cottage cheese whey different amounts of Ca$^{2+}$ and Pi were added. The samples were adjusted to different pH values by 6 N NaOH. Absorbance as turbidity of the samples was determined at 600 nm.
Addition of Ca$^{2+}$ and Pi (mMoles/L), S-1: 0 Ca$^{2+}$ + 0 Pi; S-2: 0 Ca$^{2+}$ + 19.4 Pi; S-3: 0 Ca$^{2+}$ + 38.8 Pi; S-4: 25 Ca$^{2+}$ + 0 Pi; S-5: 25 Ca$^{2+}$ + 19.4 Pi; S-6: 25 Ca$^{2+}$ + 38.8 Pi; S-7: 74.9 Ca$^{2+}$ + 0 Pi; S-8: 74.9 Ca$^{2+}$ + 19.4 Pi; S-9: 74.9 Ca$^{2+}$ + 38.8 Pi.

Fig. 3-9 Effect of pH on Colloidal Aggregation in Cheddar Cheese Whey. To 100 ml samples (from S-1 to S-9) of the defatted Cheddar cheese whey different amounts of Ca$^{2+}$ and Pi were added. The samples were adjusted to different pH values by 6 N NaOH. Absorbance as turbidity of the samples was determined at 600 nm.
Addition of Ca$^{2+}$ and Pi (mMoles/L), S-1: 0 Ca$^{2+}$ + 0 Pi; S-2: 0 Ca$^{2+}$ + 19.4 Pi; S-3: 0 Ca$^{2+}$ + 38.8 Pi; S-4: 25 Ca$^{2+}$ + 0 Pi; S-5: 25 Ca$^{2+}$ + 19.4 Pi; S-6: 25 Ca$^{2+}$ + 38.8 Pi; S-7: 74.9 Ca$^{2+}$ + 0 Pi; S-8: 74.9 Ca$^{2+}$ + 19.4 Pi; S-9: 74.9 Ca$^{2+}$ + 38.8 Pi.
Fig. 3-10 Total Protein Recovery of Precipitate by Calcium Phosphate in Cheddar Cheese Whey. To 100 ml samples of defatted Cheddar cheese whey different amounts of Ca\(^{2+}\) and Pi (mMoles/L) were added. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then the supernatants were obtained by centrifugation at 5000 x g and 30 min. The absorbances (A) at 310 nm of defatted Cheddar cheese whey and supernatants were measured by the micro-Biuret method. Recovery (%) is expressed as \(A_T / A_S\), where \(A_T = A\) of original whey and \(A_S = A\) of supernatant.

Fig. 3-11 Total Protein Recovery of Precipitation by Calcium Phosphate in Cottage Cheese Whey. To 100 ml samples of original cottage cheese whey different amounts of Ca\(^{2+}\) and Pi (mMoles/L) were added. The samples were adjusted to pH 7.5-7.8 with 6 N NaOH, then the supernatants were obtained by centrifugation at 5000 x g and 30 min. The absorbances (A) at 310 nm of original cottage cheese whey and supernatants were measured by the micro-Biuret method. Recovery (%) is expressed as \(A_T / A_S\), where \(A_T = A\) of original whey and \(A_S = A\) of supernatant.
Fig. 3-12 Scanning profiles of SDS-PAGE — comparisons for the contents of α-lactalbumin and β-lactoglobulin of the whey before and after treatment. To 100 ml samples of the original cottage cheese whey Ca$^{2+}$ and Pi were added 37.5 and 9.6 mMoles/L, respectively. To 100 ml samples of the defatted Cheddar cheese whey Ca$^{2+}$ and Pi were added 74.9 and 9.6 mMoles/L, respectively. The samples were adjusted to pH 7.5-7.8, then centrifuged at 5000 x g for 30 min to get the supernatant. Gel electrophoresis of original whey and supernatant was carried out by the procedure described in the text. All samples were loaded in 5 μl. The stained La and Lg bands were scanned by SHIMADZU dual-wavelength TLC scanner CS-910 and plotted by the integrator, LDC/Milton Roy C1-10 under the same conditions. The height of the band in original whey is designated as 100% which is compared with the height of the band in treated whey (supernatant).

(A): original cottage cheese whey;
(B): the supernatant of cottage cheese whey, La = 94.8% of original whey, Lg = 98.7% of original whey;
(C): defatted Cheddar cheese whey;
(D): the supernatant of Cheddar cheese whey, La = 95.7% of original whey, Lg = 98.9% of original whey.

La: α-lactalbumin, Lg: β-lactoglobulin.
Fig. 3-13 SDS-PAGE of samples from pretreatment of cottage cheese whey. The original cottage cheese whey was adjusted to pH 7.0 and 7.5, then centrifuged to separate the precipitate and supernatant. The precipitates from 30 ml whey were redissolved to about 2 ml by 0.1 M acetate buffer at pH 3.5. Samples of 4 μl were applied to the gel. Lane 1, MW markers; Lane 2, original cottage cheese whey; Lane 3, supernatant of pH 7.0; Lane 4, lactoperoxidase (Lp) standard; Lane 5, Pptes at pH 7.0; Lane 6, Lf standard; Lane 7, bovine serum albumin (BSA) standard; Lane 8, supernatant of pH 7.5; Lane 9, Lp standards; Lane 10, Pptes at pH 7.5; Lane 11, lactoferrin (Lf) standard; Lane 12, BSA standards.

Fig. 3-14 SDS-PAGE of samples from pretreatment of Cheddar cheese whey. The defatted Cheddar cheese whey was first adjusted to pH 4.5 by HCl then adjusted back to pH 6.5, 7.0, 7.5, 8.0 and 10.0 followed by centrifugation to separate the precipitate and supernatant. The precipitates from 30 ml whey were redissolved to about 2 ml by 0.1 M acetate buffer at pH 3.5. Samples of 4 μl were applied to the gel. Lane 1, Pptes at pH 6.5; Lane 2, Pptes at pH 7.0; Lane 3, Pptes at pH 7.5; Lane 4, Pptes at pH 8.0; Lane 5, Pptes at pH 10.0; Lane 6, bovine serum albumin (BSA) standard; Lane 7, immunoglobulin G (lgG) standard; Lane 8, lactoferrin (Lf) standard; Lane 9, defatted Cheddar whey; Lane 10, N/A; Lane 11, whole Cheddar whey; Lane 12, MW markers.
Fig. 3-15 PAS staining of SDS-PAGE — detection of glycoprotein in the extract of the precipitate by pretreatment. 1 g of precipitate powder was dissolved in 100 ml distilled water. The suspension solution was adjusted to pH 2.0 with HCl, and agitated for 20 min, and centrifuged to remove undissolved matters. The supernatant was concentrated 40 times by 10 kDa Centricon UF. 5 µl of this solution was loaded on the gel. SDS-PAGE running and PAS staining were carried out by the method described in the text.

Lane 1, lactoperoxidase standard; Lane 2, the extract of the precipitate; Lane 3, lactoferrin standard. Arrow indicates the pink band of glycoprotein by PAS staining.

Fig. 3-16 Spectrum of the extract of Precipitate with and without added Fe(NH₄)₂(SO₄)₃ (left panel) and the difference spectrum (right panel). 1 g of precipitate powder was dissolved in 100 ml distilled water. The suspension was adjusted to pH 2.0 with HCl, agitated for 20 min, and centrifuged to remove undissolved matter. The supernatant was concentrated 10 times using 30 kDa UF membrane. To 0.5 ml of the extract was added 0.5 ml 2 mM Fe(NH₄)₂(SO₄)₃ and 0.5 ml 0.1 M NaHCO₃ (curve b) or 0.5 ml water (curve a). The difference spectrum of curve b versus curve a is shown in the right panel.
Fig. 3-17 Schematic procedure of electrophoretic blotting for protein sequencing.
A 2% suspension of precipitates was adjusted to pH 2.0 with HCl, agitated for 20 min, then centrifuged to remove undissolved matter. The supernatant was ultrafiltered and diafiltered by water using a 30 kDa UF membrane. Finally, it was concentrated 10 times. Western blotting was carried out by the method described in the text.

Fig. 3-18 Effects of Ca$^{2+}$ and Pi on Iron-binding Capacity of Precipitates.
To 50 ml samples of the original cottage cheese whey was added different amounts of Ca$^{2+}$ and Pi (mMoles/L whey) and adjusted to pH 7.5 to get precipitates. All precipitates were redissolved in 30 ml and dialysed by the method described in the text. Dialysed samples were made up to 50 ml, filtered through a 0.45 µm membrane then analysed for iron-binding capacity.
Fig. 3-19 One-step Extraction of Lactoferrin by Saline Solution at Different pHs. 1% and 2% (w/v) saline solutions of lyophilized precipitates from cottage cheese whey were prepared and adjusted to pH 2.0, 6.0, 8.0 and 10.0, separately. The samples were stirred for one hr before centrifugation at 5000 x g to remove undissolved materials. The centrifuged samples were concentrated 40 times for 1% extracts and 30 times for 2% extracts by 10 kDa membrane before loading on SDS-PAGE.

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Fig. 3-20 Scanning profiles of SDS-PAGE gels for the experiments in Fig. 3-19. (A) a SDS-PAGE gel carries standard Lf and the samples extracted in 1% solution. (1) standard Lf, 1 mg/ml, (2) pH 2.0, 1% solution; (3) pH 6.0; (4) pH 8.0; (5) pH 10.0. 8 µl of standard Lf and 3 µl samples were applied to gel (A). (B) a SDS-PAGE gel carries standard Lf and the samples extracted in 2% solution. (1) standard Lf, 1 mg/ml, (2) pH 2.0, 2% solution; (3) pH 6.0; (4) pH 8.0; (5) pH 10.0. 10 µl of standard Lf and 3 µl samples were applied to gel (B). No comparison between gel (A) and (B) should be made because different parameters of the integrator were used for charting. BAFs used for 2% and 1% extracts were 8 and 7, separately. Relative % area given by the integrator is expressed as Purity. Amounts of Lf extracted are calculated by comparing the heights of the samples with that of standard Lf.
Fig. 3-21 Stepwise Extraction of Lactoferrin from Precipitates by NaH₂PO₄ at pH 6.0 and 8.0. 5 gram of lyophilized precipitates from cottage cheese whey was successively extracted by stirred with 100 ml of 0.01, 0.05, 0.1, 0.5 and 1.0 M NaH₂PO₄ solutions at both pH 6.0 and 8.0 for 20 min, followed by centrifugation at 5000 x g to remove undissolved materials. All supernatants (extracts) were concentrated 30 times using a 10 kDa membrane before loading onto the SDS-PAGE gel.

Fig. 3-22 Scanning profiles of SDS-PAGE gels for the experiments in Fig. 3-21. The profile of (S) is standard Lf in 1 mg/ml. The extracts made by NaH₂PO₄ at pH 6.0 with 0.01 M (a), 0.05 M (b), 0.1 M (c), 0.5 M (d) and 1.0 M (e). The extracts made by NaH₂PO₄ at pH 8.0 with 0.01 M (A), 0.05 M (B), 0.1 M (C), 0.5 M (D) and 1.0 M (E). 8 μl of standard Lf and 3 μl of samples were applied to the gel. Amounts of Lf extracted are calculated by comparing the heights of the samples with that of standard Lf.
Whey (Cottage and Cheddar cheese whey)

Additions of CaCl$_2$ and NaH$_2$PO$_4$ with stirring at room temperature

Adjustment of pH to 7.0-7.5 with stirring

Stirred for 10 min.

Centrifugation → Clarified whey

The precipitate paste containing Lf → Dry (Option)

The precipitate powder

Extraction of Lf by 0.5 M NaH$_2$PO$_4$ or 0.95% NaCl, pH 6.0-7.0

The extract containing Lf

UF and DF by 50 kDa membrane → Dry

CM-chromatography

Dry

Highly purified Lf

Fig. 3-23 Process for the Precipitation, Extraction and Purification of Lactoferrin from Cheese Whey

The procedures with the dotted line are optional.
Fig. 3-24  A Typical Elution Pattern of the Precipitate extract by Cation-exchange Chromatography on a Carboxymethyl (CM) ProductivTM Column. The column was equilibrated with 0.05 M acetate buffer at pH 3.7. A 30 ml extract of pH 3.7 (containing about 30 mg Lf) was loaded onto the column, then eluted with a 0-0.7 M NaCl linear gradient in 0.05 M acetate buffer at pH 3.8. The last peak indicated by the arrow was identified as lactoferrin (Lf) by SDS-PAGE.
Fig. 3.25 "In vitro" Experiments for Affinity Precipitation of Lactoferrin by Calcium and Phosphate.

(A): To 3 ml standard Lf solutions (0.3 mg Lf /ml) were added different amounts of Ca^{2+} and Pi in 9.6 mMoles/L, then adjusted to pH 7.5. The precipitates were redissolved in 3ml citrate buffer at pH 3.5. The supernatants and redissolved precipitate solutions were analysed by absorbance at 280 nm and SDS-PAGE.

(B): Photograph for SDS-PAGE gel of (A) experiments. (I) 7.5 mM Ca^{2+}; (II) 37.5 mM Ca^{2+}; (III) 74.9 mM Ca^{2+}. Lane 1, 3 and 5 are the fraction of the precipitate. Lane 2, 4, and 6 are the fraction of the supernatant.
Fig. 3-26 Hypothetical mechanism for Pseudo-Affinity Precipitation of Lactoferrin by Calcium Phosphate.

(A) Mixtures of Lf, Ca\(^{2+}\) and Pi at pH below 6.0. Ca\(^{2+}\) does not or weakly binds to Lf.

(B) Adjusted to pH 7.5 or above, Ca\(^{2+}\) first binds to Lf to form tetramer (step1), then links with phosphate (step2).

(C) Larger complex of Lf – Ca\(^{2+}\) – PO\(_4\) is formed by further cross-linking each other, which eventually precipitates.
Chapter 4 Studies on the Interaction of Lactoferrin with Calcium and Other Cations by Spectroscopic Methods

ABSTRACT

The nature of the binding of calcium to lactoferrin has been studied. Measurements by UV spectrophotometry and spectrofluorimetry show bovine lactoferrin binds 4-5 moles of calcium ion and the binding constant, pKa is 4, compared with 20 for iron (Anderson et al., 1990).

Examination of the FTIR spectra of lactoferrin (Lf) with or without cations such as Ca\(^{2+}\), Na\(^{+}\), K\(^{+}\) and Fe\(^{3+}\) have indicated that Ca\(^{2+}\) and Fe\(^{3+}\) do interact with carboxylate groups of Lf as shown by the fact that a band of apo-Lf at 1400.8 cm\(^{-1}\), arising from the O-C-O stretching vibrations of COO\(^{-}\), is apparently lost when Ca\(^{2+}\) is present or the band is shifted to give a weak shoulder near 1410 cm\(^{-1}\) with iron present. On the other hand, the spectra of Lf with Na\(^{+}\) and K\(^{+}\) do not show any change in the band near 1400 cm\(^{-1}\) as compared with apo-Lf's, suggesting that disappearance of this band in the sample of Lf with Ca\(^{2+}\) is not due to the effect of ionic strength. Protein spectra were measured in solid KBr and in deuterium oxide solutions with similar results.

It has been known (Arrondo et al., 1988) that the tyrosine band of concanavalin A in the cation-depleted form is at 1516.8 cm\(^{-1}\), whereas in the metal-binding form it is at 1515.8 cm\(^{-1}\). In this study, the samples of apo-Lf and Lf with Ca\(^{2+}\) show a band at 1516.1 cm\(^{-1}\), on the other hand, the sample of Lf with Fe\(^{3+}\) gave a band at 1515.1 cm\(^{-1}\). The result clearly indicates that tyrosine is involved in binding iron but not in calcium.

The sensitive amide I' band (1700-1620 cm\(^{-1}\)) has been used for analysis of the secondary structure of lactoferrin as well as detection of possible changes due to interactions of metal ions. The data have indicated that in the presence of Ca\(^{2+}\), the amount of the secondary structures of lactoferrin such as \(\alpha\)-helix, \(\beta\)-sheet, turns etc., have undergone a great alteration whereas K\(^{+}\) and Na\(^{+}\) have caused a small change. On the contrary, Fe\(^{3+}\) gave almost no change in \(\alpha\)-helix and \(\beta\)-sheet content of lactoferrin. Ca\(^{2+}\) exerted the greatest change in \(\alpha\)-helix and \(\beta\)-sheet content of lactoferrin with a decrease of 27-46% and an increase of 19-23%, respectively.

A previous study indicated that the lactoferrin region involved in the binding with animal or microbial cells is the sequence of residues 4-52 on the N-lobe of domain-I with an \(\alpha\)-\(\beta\)-\(\alpha\)-\(\beta\) secondary structure (Spik et al., 1993). Ca\(^{2+}\)-induced changes of the \(\alpha\)-helix and \(\beta\)-sheet in the lactoferrin structure might be responsible for the experimental observations that Ca\(^{2+}\) ions reduce or reverse the antimicrobial capability of lactoferrin (Ellison et al., 1990; Ellison & Giehl, 1991).
4.1 INTRODUCTION

The transferrins are a family of monomeric glycoproteins with molecular weights of about 80,000 dalton that are able to bind two ions of Fe$^{3+}$ per molecule (Haden et al., 1994). The family includes lactoferrin (Lf), serum transferrin (sTf) and ovotransferrin (OTs) and member proteins are widely distributed in the physiological fluids of vertebrates (Aisen & Listowsky, 1980). Despite the similarity, serum transferrin and lactoferrin have different physiological roles. Serum transferrin is mainly involved in the transport and donation of iron to cells. This process involves complexing of iron by the transferrin, then the transferrin-iron complex binds to a cell surface receptor, endocytosis, acidification (pH 5-6), release of iron and recycling of the complex to the cell surface where the molecules dissociate and repeat the cycle (Haden et al., 1994). Lactoferrin, on the other hand, plays a role in the defense against bacteria that require iron to survive (Norris et al., 1989) or in directly damaging outer membranes of Gram-negative bacteria (Ellison et al., 1990). Lactoferrin is also involved in many other biological functions (Brock et al., 1993). Although lactoferrin is widely regarded as an iron-binding protein, a notable characteristic is its diversified binding ability. Human lactoferrin was reported to interact with casein and albumin (Hekman, 1971), secretory IgA (Watanabe et al., 1984), lysozyme (Jorieux et al., 1985), β-lactoglobulin (Lampreave et al., 1990), and receptors of animal (Pierce et al., 1991) and microbial cells (Pettersson & Tommassen, 1994). In 1981 Bennett et al. (1981) reported that Lf can self associate forming tetramers in the presence of calcium, after which inhibition of granulocyte colony-stimulating activity of Lf is lost. Other
reports (Ellison et al., 1990; Ellison & Giehl, 1991) indicated that the ability of lactoferrin to damage the Gram-negative outer membrane by lipopolysaccharide release can be blocked by addition of Ca$^{2+}$. However, it is quite curious that human lactoferrin (HLf) was found not to have a significant ability to chelate calcium in an equilibrium dialysis study (Ellison & Giehl, 1991). Although the properties of BLf are similar to those of HLF (Spik et al., 1993), such as the bilobal tertiary molecule, characteristic iron-binding and identical secondary structure (α-β-α-β structure) in amino acid residues 1-52, the sequences of Blf and HLF have only 69% homology (Spik et al., 1993). Thus the 31% difference between primary structures probably shows the differences in the properties of BLf and HLF.

All proteins (Bagshaw & Harris, 1987) contain natural chromophores absorbing in the near ultraviolet, notably the aromatic residues phenylalanine, tyrosine, and tryptophan ($\lambda_{\text{max}} = 260-290$ nm). These chromophores are also responsible for protein fluorescence. In most proteins tryptophan fluoresces ($\lambda_{\text{emit}} = 340$ nm), light absorbed by other aromatic residues being transferred to tryptophan non-radiatively. Absorbance and/or fluorescence of these residues may change when protein structure is perturbed by binding with other species. Absorbance changes are typically small but may be observable, particularly if tyrosine is transferred from an apolar to a polar environment. Protein fluorescence changes, which are more sensitive, are commonly used to measure species binding to proteins (Bagshaw & Harris, 1987). These techniques have been successfully used to study binding of Ca$^{2+}$ to α-lactalbumin (Kronman et al., 1981).
Numerous physical techniques exist for the structural analysis of ligand-binding sites and in consequence conformational changes in proteins. Of these, the most widely used are X-ray diffraction, nuclear magnetic resonance (NMR), circular dichroism (CD) spectroscopy and Fourier transform infrared (FTIR) spectroscopy. Each technique, however, has its own particular problems (Jackson et al., 1989; Jackson & Mantsch, 1995). X-ray diffraction requires the production of relatively large (200 mm) crystals which are sometimes difficult to obtain. CD spectroscopy is unable to distinguish between β-pleated sheet and turn structures, and the spectra often contain overlapping contributions from amino acid side chains. Owing to line broadening effects, NMR spectroscopy can only presently be applied to proteins with a molecular weight of less than 20,000 Dalton, and it cannot readily applied to the study of proteins in a membrane environment. Therefore, it can readily be seen that there are many instances where FTIR spectroscopy may be only one of many methods available for characterization (Jackson & Mantsch, 1995).

X-ray crystal structures have been reported for human lactoferrin in its diferric (Anderson et al., 1989) and apo-forms (Anderson et al., 1990), whereas a similar study on bovine lactoferrin is in progress (Baker & Lindley, 1992). X-ray data have revealed that human lactoferrin has a bilobal structure. The protein is folded into two equal size lobes with very similar structure. The two lobes comprise residues 1-332 (N-lobe) and 344-703 (C-lobe). N- and C-lobes are divided into 4 domains (N1, N2, C1 and C2), where the iron-binding sites are situated in the interdomain cleft with iron taking ligands from both domains. Protein ligands (Baker et al., 1987), which are the same in
both sites are Asp61, Tyr93, Tyr191 and His252 (Asp407, Tyr447, Tyr540 and His609 are in the C-lobe).

The structural changes involved in the binding of metal ions, particularly Ca\(^{2+}\), to a number of proteins have been investigated using FTIR spectroscopy. Changes in the structure of the concanavalin A associated with its “demetallisation” and methylmannose binding were shown using FTIR spectroscopy (Alvarez et al., 1987). In the presence of Ca\(^{2+}\) and Mg\(^{2+}\), the amide II band of apo concanavalin A at 1532 cm\(^{-1}\) shifts 4-6 cm\(^{-1}\) to higher wavenumbers, and the amide I component at 1634 shifts 1 cm\(^{-1}\) in the same direction, both in H\(_2\)O and D\(_2\)O buffer, suggesting changes in the hydrogen-bonding network of a large portion of protein, particularly in the β-sheet regions. Effects of Ca\(^{2+}\) and Mg\(^{2+}\) binding for calmodulin and troponin C structures have been studied, where FTIR spectroscopy was used to examine the conformation sensitive amide I bands of proteins (Trewhella et al., 1989). A decrease in intensity at 1654 cm\(^{-1}\), the normal frequency assignment for α-helical structure, is observed as Ca\(^{2+}\) binds to calmodulin and troponin C. This suggests that Ca\(^{2+}\) binding results in a net decrease in "normal" α-helix conformation. Examination of FTIR spectra of lyophilized whole casein with and without Ca\(^{2+}\) ions provides direct evidence for the interaction between these divalent ions and the carboxylate groups of glutamate and aspartate (Byler & Farrell, 1989). In the absence of Ca\(^{2+}\), the O-C-O stretching vibrations of these carboxylates give two characteristic infrared bands near 1400 and 1575 cm\(^{-1}\). When Ca\(^{2+}\) ions are present, this pair of bands was observed to shift about 10 cm\(^{-1}\) to approximately 1410 and 1565 cm\(^{-1}\), respectively. In addition, FTIR was also used to investigate secondary structures of human serum transferrin, human lactoferrin
and rabbit serum transferrin in their diferric and apo forms (Haden et al., 1994). This study has shown that: (A) The secondary structure of all the proteins studied in H₂O was in the range 43-53% α-helix and 23-28% β-sheet. (B) The secondary structural content of the proteins is not altered by iron binding or release. However, the iron-free proteins undergo a greater extent of $^1$H - $^2$H exchange than the diferric proteins indicating that significant structural changes do occur upon iron binding/release.

In this study, the interactions of bovine lactoferrin with calcium ion and other cations were investigated by more sensitive methods, including spectrophotometry, spectrofluorimetry and Fourier transform infrared spectroscopy (FTIR).

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Apo-lactoferrin

Very pure lactoferrin samples with iron saturation of 68% was kindly donated by Dr Regester, Melbourne Laboratory, CSIRO Division of Food Science & Technology. Bovine apo-lactoferrin (apo-Lf) was obtained by dialysis using a modified method of Masson & Heremans (1968). The lactoferrin (Lf) sample was dissolved in MilliQ distilled water with 40 mM EDTA and placed in Spectrapor membrane tubing with MW cutoff 6000-8000, then dialysed against 20 volumes of the 0.1 M citrate buffer pH 4.0 at 4°C for 36 hr (renewed once) to remove the bound iron. The citrate was then eliminated by dialysis against 20 volumes of deionized water (twice renewed) for 24 hr at 4°C. Dialysed apo-Lf solution was added to 4 volumes of MilliQ water and diafiltered twice using an Amicon disc ultrafilter with 30 kDa cut-off membrane
(YM30) in a further effort to remove as much contamination as possible before concentration and lyophilization to yield dry apo-Lf. No α-Lactalbumin was detected by electrophoresis in the apo-Lf sample even at very high loading (20 μg total protein). The identity of the apo-Lf sample was confirmed by sequencing the N-terminus to residue 10 on an Applied Biosystems 470A Protein Sequencer. The resultant sequence “1 A P R K N V R W C T 10” is identical with that of Lf (Bos Primigenius Taurus) analysed by Pierce et al. (1991).

4.2.2 Titration of Lactoferrin with Ca$^{2+}$

(A) Ligand binding of Ca$^{2+}$ by Lf measured by spectrophotometry and spectrofluorimetry

Difference spectra of samples of apo-Lf and lactoferrin with calcium (Ca-Lf) were recorded by Perkin Elmer Lambda 2 UV/Vis spectrophotometer equipped with software PECSS version 3.2.

The fluorimeter used was an Aminco-Bowman Series 2, luminescence spectrometer with an OS/2-PM operating system. Excitation wavelength of 290 nm was used and the emission spectra were measured from 300 to 430 nm.

(B) Determination of Apparent Association Constant ($K_{ass}$) by Titration at 280nm

Apparent association constant ($K_{ass}$) was determined by using the mathematical treatment proposed by Segel (1976). In enzyme kinetics, the Henri-Michaelis-Menten equation in rapid equilibrium approach can be written as:

$$\frac{v}{V_{max}} = \frac{[S]}{(K_s + [S])}$$  \hfill (4-1)

where $K_s$ is dissociation constant, $v$ is instantaneous velocity, $V_{max}$ is the maximal velocity, $[S]$ is the concentration of substrate.
\( K_s \) can obtained by a Scatchard plot of the Henri-Michaelis-Menten equation:

\[
\frac{v}{[S]} = - \frac{v}{K_s} + \frac{V_{\text{max}}}{K_s}
\]  

(4-2)

The binding of ligand to protein can be also analyzed by this rapid equilibrium approach (Segel, 1976). In this study, the binding of calcium (Ca) by lactoferrin (Lf) is given:

\[ [\text{Ca}]_b = [\text{Lf} \text{ Ca}] = \text{the concentration of bound Ca} \]

\[ [\text{Ca}]_r = \text{the concentration of free Ca} \]

\[ [\text{Lf}]_t = \text{the total lactoferrin concentration} \]

\[ n \ [\text{Lf}]_t = \text{the total concentration of binding sites in lactoferrin} \]

The modification (Segel, 1976) involves substituting \([\text{Ca}]_b \) for \( v \), and \( n \ [\text{Lf}]_t \) for \( V_{\text{max}} \) since:

\[ v \propto [\text{Ca}]_b \quad \text{and} \quad V_{\text{max}} \propto n \ [\text{Lf}]_t \]

The equation becomes:

\[
[\text{Ca}]_b / [\text{Ca}]_r = - [\text{Ca}]_b / K_s + n \ [\text{Lf}]_t / K_s
\]  

(4-3)

Since association constant \( K_{\text{ass}} \) equals \( 1 / K_s \), the equation (4-3) can be rewritten as follows:

\[
[\text{Ca}]_b / [\text{Ca}]_r = - K_{\text{ass}} \cdot [\text{Ca}]_b + n \ [\text{Lf}]_t \cdot K_{\text{ass}}
\]  

(4-4)

\( K_{\text{ass}} = \text{Slope,} \quad n = \text{Intercept} / [\text{Lf}]_t \cdot K_{\text{ass}}. \)

For the titration of Lf, two tandem cells, in the reference and sample beams, respectively, each contained 2.5 ml of 12.5 \( \mu \text{M} \) Lf solution ( \([\text{Lf}]_t \) ) prepared with 0.05 M Tris buffer at pH 7.5 or 0.05 M acetate buffer at pH 4.5, and gave an absorbance of 0.0000 - 0.0002 at 280 nm. A 2 \( \mu \text{l} \) aliquot of 1.0 M \( \text{CaCl}_2 \) in 0.05 M Tris buffer, pH
7.5 or 0.05 M acetate buffer at pH 4.5 was added to the sample cell each time and the
differences in absorbance (ΔA) at 280 nm were recorded until stable. ΔA and added
Ca$^{2+}$ were equivalent to [Ca]$_b$ and [Ca]$_f$, thus ΔA/added Ca$^{2+}$ versus ΔA was plotted
as [Ca]$_b$/[Ca]$_f$ versus [Ca]$_b$, then $K_{ass}$ and n were computed by the least square
method.

4.2.3 FTIR Measurements

(A) Preparation of Samples for FTIR Examination

For solid samples, apo-lactoferrin was first dissolved in D$_2$O (deuterium oxide,
99.9%, Sigma Chemicals) and left overnight to obtain maximum exchange of hydrogen
with deuterium. Five D$_2$O solutions of samples, namely (a) Apo-Lf: 10 mg apo-
lactoferrin at pD 6.8, (b) Ca-Lf: 10 mg apo-Lf + 1.5mM CaCl$_2$/g Lf at pD 7.5, (c) K-
Lf: 10 mg apo-Lf + 4.5 mM Kcl/g Lf at pD 7.5, (d) Fe-Lf: apo-Lf + 1.5 mM
FeCl$_3$/g Lf at pD 7.5, (e) Na-Lf: 10 mg apo-Lf + 4.5 mM NaCl/g Lf at pD 7.5, were
prepared then lyophilized again. pD values of the solutions were adjusted by 1.0 M
DCl and 1.0 M NaOD, which were prepared by dissolving reagent grade concentrated
HCl and NaOH pellets in D$_2$O (Timasheff, 1972). Finally, all solutions were
lyophilized to get dry lactoferrin/cation mixtures. KBr pellets of all solid samples were
prepared by mixing 300 mg KBr and 2 mg of each lactoferrin/cation mixture which
was then pressed for several minutes in a hydraulic press by applying between 8 and 20
tons/cm$^2$ pressure.

For liquid samples, apo-Lf was dissolved in D$_2$O at a concentration of 32.5
mg/ml, and two D$_2$O solutions were prepared as, (1) Apo-Lf : apo-Lf at pD 6.8, (2)
Ca-Lf : apo-Lf + 1.5 mM CaCl₂/g Lf at pH 7.3. pH values of the solutions were adjusted by the method mentioned above (Timasheff, 1972).

(B) Infrared Spectra

Transmission FTIR spectra of samples as both KBr pellets and D₂O solutions were recorded with a BioRad FTS7 FTIR spectrometer (BioRad Lab., Digilab Division, Cambridge, Mass.) equipped with HgCdTe and MCT detectors and a germanium-potassium bromide beamsplitter. A BioRad Model FTS7 3200 data station was used for data acquisition, storage, and analysis. The samples were scanned from 4000 cm⁻¹ to 400 cm⁻¹ with resolutions at both 2 cm⁻¹ and 8 cm⁻¹ for aqueous solution and solid samples, respectively. The aqueous solution was placed between two CaF₂ windows that were assembled into a demountable infrared cell. Potential contamination of the sample by dissolution of Ca²⁺ from CaF₂ window was ruled out by Trehwella et al. (1989). The thickness of the spacer was 12 μm for samples in D₂O.

Spectral deconvolution and second derivative treatment were performed using the BioRad Fourier Manipulations software. The deconvolution parameters, σ = 20, K = 2.2 were used for solid samples and σ = 6.5, K = 2.0 for liquid samples. Quantitative analysis of the secondary structures of apo-Lf and ligand-Lf was achieved using the BioRad 'Bandfit' curve fitting programme. When the components of the deconvoluted spectrum in amide I' band (1700 - 1620 cm⁻¹) were fitted using the 'Bandfit' programme, the relative areas of the components give the percentage contribution of each component to the amide I' bands, which correspond to the secondary structure of proteins. Because of the irregular shape of the deconvoluted curves, automatic and manual (joystick) optimizations were intermingled to make the
RMS deviation of 'Bandfit' a minimum (the smaller value the RMS deviation is, the better the fit). The RMS convergence factor of BioRad "Bandfit" is 0.001. RMS values were obtained as 0.04 - 0.07 for optimizations of KBr pellets, and 0.01-0.02 for D2O solutions of samples. Reproducibility of the quantitative analysis procedure was estimated by the following method: Two liquid Ca-Lf samples with a concentration of 32.5 mg/ml were scanned with the FTIR spectrometer, separately, then were subjected to deconvolution, "Bandfit" treatment, and percentage calculation of α-helices (α), β-structures (β), unordered structure (U), and turns (T). The secondary structures obtained are α1 = 20.4%, α2 = 23.6%, β1 = 39.3%, β2 = 45.0%, U1 = 15.5%, U2 = 9.9%, T1 = 24.6%, T2 = 20.6%. Standard Deviations (σ) are calculated as σα = 2.3, σβ = 4.0, σU = 4.0, and σT = 3.0.

4.3 RESULTS AND DISCUSSION

4.3.1 Measurements of Ca-ligated Lactoferrin by Spectrophotometry and fluorimetry

Addition of Ca²⁺ to apo-Lf had a significant effect on its molecular conformation, as shown by the absorption difference spectrum (Fig. 4-1) and tryptophan emission spectrum (Fig. 4-2). Similar effects were also seen when Ca²⁺ interacted with bovine α-lactalbumin (Kronman et al., 1981). In the difference spectra of Ca-Lf against apo-Lf, a significant absorption peak of Ca-Lf at pH 7.5 (Fig. 4-1, II) was recorded around 280 nm as compared with little absorption of Ca-Lf at pH 4.5 as shown in Fig. 4-1(I). Such an enhanced absorption by addition of Ca²⁺ to Lf implies a
structural change caused by Ca\(^{2+}\) binding to Lf. However, the affinity of Ca\(^{2+}\) for Lf was dramatically reduced at low pH (pH 4.5), suggesting that Ca\(^{2+}\) formed a complex with Lf that was dissociated at pH 4.5.

The intrinsic fluorescence emission spectra of apo- and Ca-Lf have given further evidence that Ca\(^{2+}\) and Lf formed complexes at pH 7.5 (Fig. 4-2). This figure shows a profound difference in the tryptophan emission spectra for apo-Lf and Ca-Lf. A clear increase in fluorescence intensity was monitored at 335 nm. In addition, a subtle change in the structure of apo-Lf and Ca-Lf curves was distinguished, where the spectrum of Ca-Lf contained many more small and overlapping peaks which imply structural change by Ca\(^{2+}\) binding to Lf.

The association constant for Ca\(^{2+}\) binding to Lf, K\(_{ass}\), was determined by a direct calcium titration using absorption difference (ΔA) at 280 nm in this study. This method had been used for the titration of transferrin by the metal ions (Gelb & Harris, 1980).

The titration curve for Ca\(^{2+}\) and Lf is shown in Fig. 4-3, where ΔA at 280 nm is plotted against the additions of Ca\(^{2+}\) ions. Titration at pH 7.5 gave a curve in which ΔA was progressively increasing aliquot by aliquot of Ca\(^{2+}\) added until approaching a plateau. On the other hand, a flat curve was obtained by titration at pH 4.5. A Scatchard plot of the titration curve at pH 7.5 was plotted using the regression programme of Excel (Fig. 4-4). Thus K\(_{ass}\), the slope of the Scatchard plot, was approximately 1.98 x 10\(^{4}\) M\(^{-1}\) while the number of binding sites (n = 5) was calculated
from the intercept \((C_a)\). Table 4-1 has listed \(K_{ass}\) and \(n\) of some proteins binding calcium.

### 4.3.2 FTIR Examinations of Apo-lactoferrin and Metal-ligated Lactoferrin

Usually, studies of interactions between proteins and ligands are undertaken to shed light on a number of intriguing questions including (a) side chains (amino acid residues) in the protein responsible for ligand-binding, (b) conformational changes of the protein following ligands-binding. In infrared spectra, the answers for these two aspects can be interpreted from vibrations of the groups of peptide bond (C=O, NH, CN) in the protein backbone which are designated as Amide bands, and vibrations of characteristic groups of side chains in the protein.

The most useful bands for the infrared study of conformational changes of proteins are the amide I bands approximately between 1700 - 1600 cm\(^{-1}\) (Susi, 1972). In practice amide I' is used in the region of 1700 - 1620 cm\(^{-1}\). Band assignments in amide I', which give the analysis of secondary structure of protein such as \(\alpha\)-helix, \(\beta\)-structures and turns, have been well established (Byler & Susi, 1986; Susi & Byler, 1986; Byler & Susi, 1988). Although attempts have also been made to utilize other vibrational modes, particularly the amide II (1480-1575 cm\(^{-1}\)) and amide III (1229-1301 cm\(^{-1}\)), assignments for amide II and III bands are still not well established. Band assignments for protein side-chains are even more difficult because many side-chain vibrations overlap with amide bands. Fortunately, there are still some "amide-irrelevant" bands that can be used for interpretation of side-chains of the protein.

It can be inferred that anionic side chains in the protein, e.g. carboxylate groups of aspartate and glutamate, phenolic groups of tyrosine, have a strong preference for
binding cations with a high positive charge. The iron binding sites of human Lf have been identified as Asp61, Tyr93, Tyr191 and His252 in the N-lobe, and Asp407, Tyr447, Tyr540 and His609 in the C-lobe, respectively (Baker et al., 1987). The calcium binding sites of α-lactalbumin, on the other hand, have been revealed as Asp82, Asp84, Asp87 and Asp88 (Stuart et al., 1986). So, carboxylate anion and phenol groups of Lf side-chains are likely positions to ligate calcium ion.

The carboxylate anion gives rise to two bands (Silverstein et al., 1992): a strong asymmetrical stretching band near 1650-1550 cm\(^{-1}\) and a weaker, symmetrical stretching band near 1400 cm\(^{-1}\). Because the frequencies 1650-1550 cm\(^{-1}\) partially overlap with amide I and II, the band between 1560 and 1585 is independent, and can be assigned to asymmetrical stretching vibration of carboxylate groups (Timasheff, 1972). The symmetrical stretching band near 1400 cm\(^{-1}\) (approx. 1420-1330 cm\(^{-1}\)) is completely independent of any amide and side-chain bands, and was satisfactorily used to identify carboxylate group (Byler & Farrell, 1989).

A band due to the C-C stretching vibration of the tyrosine aromatic ring was assigned in the study of concanavalin A (Arrondo et al., 1988). For native concanavalin A (containing cations), the maximum of the tyrosine band is at 1515.8 cm\(^{-1}\), whereas in the cation-depleted protein it is at 1516.8 cm\(^{-1}\) and in the aggregated protein at 1512.2 cm\(^{-1}\).

The original spectra of samples of apo-lactoferrin (apo-Lf), lactoferrins with Ca\(^{2+}\) (Ca-Lf), K\(^{+}\) (K-Lf), Na\(^{+}\) (Na-Lf) and Fe\(^{3+}\) (Fe-Lf) are shown in Fig. 4-5 in the region of 4000-400 cm\(^{-1}\) and Fig. 4-6 in the region of 1700-1200 cm\(^{-1}\). Bovine lactoferrin gives typical absorptions in amide A and B (\(\sim\)3100 cm\(^{-1}\)), amide I (1700-
1600 cm\(^{-1}\)) and amide II (1575-1480 cm\(^{-1}\)) regions according to Fig. 4-5. In Fig. 4-6 the spectra of different samples have been focused on in the region of 1700-1200 cm\(^{-1}\) which is most interesting for the study of protein. Few differences among the original spectra of these samples are obvious, except for a band of apo-Lf (Fig. 4-6, I) at 1400.8 cm\(^{-1}\) which has apparently been lost in the presence of calcium (Fig. 4-6, G) or became a shifted weak shoulder near 1410 cm\(^{-1}\) in the presence of iron (Fig. 4-6, F). However, the spectrum of lactoferrins with Na\(^+\) (Fig. 4-6, H) and K\(^+\) (Fig. 4-6, J) did not show any change in the band near 1400 cm\(^{-1}\) as compared with apo-Lf’s (Fig. 4-6, I), suggesting that disappearance of a band at 1400 cm\(^{-1}\) in the sample Ca-Lf is not due to the effect of ionic strength but to interaction between side chains of lactoferrin and calcium. Second derivative spectra with higher resolution have further confirmed that the bands of Na-Lf (Fig. 4-7, a), apo-Lf (Fig. 4-7, b) and K-Lf (Fig. 4-7, c) are highly similar except for amide I bands (1700-1600 cm\(^{-1}\)), suggesting that ionic strength will influence backbone conformation other than the side-chains of lactoferrin.

As already mentioned, when the original spectrum of Fe-Lf was compared with apo-Lf and Ca-Lf (Fig. 4-6), the band near 1400 cm\(^{-1}\) was shifted in Fe-Lf, but the band almost disappeared in Ca-Lf. The evidence suggests that more carboxylate groups of Lf are involved in interaction with calcium than with Fe\(^{3+}\). It has been well known that two molecules of Asp in human lactoferrin (one each lobe) are responsible for binding iron (Baker et al., 1987).

After the original spectra were differentiated, two sharp peaks of apo-Lf at 1452.0 and 1397.1 cm\(^{-1}\) have been obtained in second derivative spectra (dots, in Figs. 4-8, 4-9). By comparison in Fig. 4-8, the band at 1397.1 cm\(^{-1}\) in apo-Lf spectrum
(dots) was shifted to higher frequency at 1408.8 cm\(^{-1}\) in Ca-Lf's (line) with significantly reduced intensity (65%), and the spectrum of Fe-Lf (line, in Fig. 4-9) by contrast has a shift of the band at 1397.1 cm\(^{-1}\) to lower frequency at 1391.7 cm\(^{-1}\) with intensity only 18% reduced. In addition, second derivative spectra of both Ca-Lf and Fe-Lf have given two negative bands at 1385.3 cm\(^{-1}\) and 1373.3 cm\(^{-1}\) that are apparently created from the band 1397.1 cm\(^{-1}\) of apo-Lf by addition of Ca\(^{2+}\) and Fe\(^{3+}\). As it is known that O-C-O stretching vibrations of carboxylates gives rise to a band near 1400 cm\(^{-1}\) (Silverstein et al., 1992), such shifts or changes of this band in lactoferrin with Ca\(^{2+}\) and Fe\(^{3+}\) suggest that interaction or binding between carboxylate groups and these metal ions, Ca\(^{2+}\) and Fe\(^{3+}\), occurs.

The spectra of apo-Lf and Ca-Lf in aqueous solutions with D\(_2\)O have been shown in Fig. 4-10 (original) and Fig. 4-11 (second derivative). In the presence of calcium (Fig. 4-10, d), an original band at 1401.6 cm\(^{-1}\) of apo-Lf (Fig. 4-10, e) disappeared. In second derivative spectra (Fig. 4-11), the band at 1401.6 cm\(^{-1}\) of apo-Lf (dots) becomes two very weak bands on addition of Ca\(^{2+}\) (line) relative to a negative one at 1404.2 cm\(^{-1}\), and a positive one at 1399.2 cm\(^{-1}\). These data have further confirmed that calcium is sure to interact with carboxylate groups of lactoferrin in solid or liquid media.

In the region of approx. 1480-1370 cm\(^{-1}\), all the original spectra of apo-Lf in both solid and liquid states have shown two peaks, one near 1450 cm\(^{-1}\), and the other near 1400 cm\(^{-1}\) (see Figs. 4-6, 4-10). It has been discussed above that the band at 1400 cm\(^{-1}\) designates the carboxylate anion group, and this band will be altered in the
presence of Ca\(^{2+}\) and Fe\(^{3+}\). However, the band assignment of 1450 cm\(^{-1}\) remains unknown. Because O-H bending vibration of the carboxylic acids is referred to 1440-1395 cm\(^{-1}\) (Silverstein et al., 1992), the bands at 1450 cm\(^{-1}\) are probably associated with the carboxylic acid form, e.g. COOH. In the original spectra in Figs.4-6, 4-10, it has been observed that with Ca\(^{2+}\) and Fe\(^{3+}\) present the band at 1400 cm\(^{-1}\) disappeared and the intensity of the band at 1450 cm\(^{-1}\) increased (1400 cm\(^{-1}\) being moved and piled up to 1450 cm\(^{-1}\)), while in apo-Lf two bands at both 1400 and 1450 cm\(^{-1}\) have similar intensities. The band position and absorbance intensity (Table 4-2) calculated from Fig. 4-6 and Fig. 4-10 have indicated that with Ca\(^{2+}\) and Fe\(^{3+}\) present the band near 1400 cm\(^{-1}\) disappeared and the intensity of the band near 1450 cm\(^{-1}\) increased (assuming that 1400 cm\(^{-1}\) is moved and piled up to 1450 cm\(^{-1}\)), while in apo-Lf two bands at both 1400 and 1450 cm\(^{-1}\) have similar intensities. From this observation it has been inferred that on the one hand the carboxylic groups in apo-Lf at natural pH (about 6.8) exist as two forms COO\(^{-}\) and COOH, assigned to the bands near 1400 and 1450 cm\(^{-1}\), respectively, on the other hand the carboxylic groups in Ca-Lf and Fe-Lf at pH 7.5 become metal-binding forms, COOCa and COOFe which are also assigned to the band near 1450 cm\(^{-1}\). So, a shift of the band at 1400 cm\(^{-1}\) to the band at 1450 cm\(^{-1}\) in FTIR spectra might be interpreted as titration of the carboxylate anion side-chain in lactoferrin by H\(^{+}\), Fe\(^{3+}\) and Ca\(^{2+}\) as follows:

\[
\text{COO}^{-} + \text{H}^{+} \rightleftharpoons \text{R-COOH}
\]

\[
\text{R-COO}^{-} + \text{Ca (Fe)} \rightleftharpoons \text{R-COO Ca (Fe)}
\]

The appearance of two bands near 1450 and 1400 cm\(^{-1}\) with similar absorbance supports the notion that equilibrium between COOH and COO\(^{-}\) in apo-lactoferrin is
reached (Table 4-2). When metal ions, eg Ca\(^{2+}\) or Fe\(^{3+}\), are added, carboxylate anions of apo-Lf bind with metal ions, resulting in disappearance of the band near 1400 cm\(^{-1}\) (Fig.4-6 G vs. I and 4-10 d vs.e) or its displacement to a shoulder (Fig.4-6 F vs. I). Because carboxylate anions are changed to salt forms on binding metal ion, the intensities of the bands assigned to both COOH and COOCa(Fe) near 1450 cm\(^{-1}\) are increased (Figs. 4-6 F, G, 4-10 d).

In addition, second derivative spectra have shown that the band of apo-Lf at 1452 cm\(^{-1}\) has created two partially resolved peaks (dots in Figs. 4-8, 4-9); with Ca\(^{2+}\) present one is 1463.0 cm\(^{-1}\) and the other 1450.5 cm\(^{-1}\) as compared with 1461.1 and 1435.7 cm\(^{-1}\) in presence of Fe\(^{3+}\). This band alteration with metal ions present could imply fine structures of carboxylate binding with metal ions. So far, all the evidence strongly suggests that carboxylate groups are involved in interacting with Ca\(^{2+}\) and Fe\(^{3+}\), and furthermore supports claims that lactoferrin can bind calcium.

Tyrosine residues of concanavalin A have shown a FTIR band at 1516.8 cm\(^{-1}\) in the apo-form but a shift to 1515.8 cm\(^{-1}\) in the metal-binding form because of a change in the C-C stretching vibration of the tyrosine aromatic ring (Arrondo et al., 1988). In the present study, all the apo-Lf, Ca-Lf as well as Ca-Lf in D\(_2\)O solution give the same band at 1516.1 cm\(^{-1}\) (Fig. 4-12 a,b,c) whereas Fe-Lf makes that band shift to 1515.1 cm\(^{-1}\) (Fig. 4-12 d). This result clearly indicates that Tyr is involved in binding iron but not in calcium. Since according to X-ray crystallography each iron binding site of human lactoferrin contains two residues of Tyr (Baker et al., 1987), it is judged that there are two possible sites binding calcium. One is that Ca\(^{2+}\) binding site is different from that binding iron, furthermore, calcium is not bound into the
interdomain cleft with side chains from both domains. Another is that Ca could be at the same site with Fe but not bound to tyrosine there.

As shown above, FTIR spectral changes concerned with carboxylate binding Ca$^{2+}$ or Fe$^{3+}$, are so different as to indicate a difference in the metal-binding structure including types (Glu or Asp), positions and the coordination geometries of carboxylate groups, should be considered. Unfortunately, the FTIR technique cannot answer the first two questions but it can infer the geometrical structure of carboxylate ligand.

A carboxylate ion, RCOO$^-$, can coordinate to metals in a number of ways, viz. as a unidentate ligand (I), as a chelating (bidentate) ligand (II), as a bridging bidentate ligand (Deacon & Phillips, 1980). Examples of the various arrangements are given below.

Unidentate coordination [I] removes the equivalent of the two oxygen atom (Nakamoto, 1963). If the carbon-oxygen bond is appreciably affected, a pseudo-ester configuration (see [I]) is obtained. This should increase $\nu_{\text{sym}}$(CO$_2$)*, decrease $\nu_{\text{asym}}$(CO$_2$)* and increase the separation ($\Delta^*$) between the $\nu$(CO2) frequencies (wavenumbers) relative to values for the free carboxylate ion (Nakamoto, 1963). In other words, larger $\Delta^*$ values for carboxylate complexes appear generally to be associated with unidentate coordination.
Chelation ([III]) or symmetrical bridging ([III]) should not alter the bond orders, and it is also suggested that symmetrical bridging or chelation (Deacon & Phillips, 1980) shifts both $v_{\text{asym}}(\text{CO}_2)^*$ and $v_{\text{asym}}(\text{CO}_2)^*$ frequencies in the same direction. Thus, smaller $\Delta^*$ values for an acetate complex is generally indicative of chelation or of an acetate group that is both chelating and bridging (Nakamoto, 1963).

Byler and Farrel have used the difference in band positions between two original spectra to infer the geometrical structure of carboxylate-Ca$^{2+}$ in whole casein (Byler & Farrell, 1989). When the spectrum of casein without Ca$^{2+}$ was subtracted from the spectrum of casein with Ca$^{2+}$, the difference bands with negative intensity at 1581 and 1393 cm$^{-1}$ were considered to be $v_{\text{asym}}$ and $v_{\text{sym}}$ of carboxylate anion, respectively, and the positive difference bands at 1560 and 1423 cm$^{-1}$ probably correspond to $v_{\text{asym}}$ and $v_{\text{asym}}$. It is obvious that the difference ($\Delta$) without Ca$^{2+}$ (188 cm$^{-1}$) is higher than $\Delta$ with Ca$^{2+}$ (173 cm$^{-1}$), consequently bidentate binding mode [II] of carboxylate-Ca$^{2+}$ was proposed for casein (Byler & Farrell, 1989).

When this method was used in this study, the difference spectrum of Ca-Lf minus apo-Lf (Fig. 4-13) also gives two negative bands at near 1540 and 1392 cm$^{-1}$ considered as $v_{\text{asym}}$ and $v_{\text{asym}}$ of apo-Lf, and two positive bands at 1560 (a shoulder) and 1450 cm$^{-1}$ as $v_{\text{asym}}$ and $v_{\text{asym}}$. Thus, $\Delta_{\text{apo-Lf}}$ was calculated as 148 cm$^{-1}$, more than 110 cm$^{-1}$ of $\Delta_{\text{Ca-Lf}}$. In addition, both $v_{\text{asym}}(\text{CO}_2)$ and $v_{\text{asym}}(\text{CO}_2)$ wavenumbers are shifted to higher values in the same direction. These data imply that the binding of Ca-carboxylate is either chelation [II] or bridging [III] and similar to that suggested by Byler and Farrel (1989).
Calcium and iron typically exhibit approximately octahedral coordination. For simplicity, structures I-III schematically depict only one or two of the six coordination sites on the metal ion (M). In a protein, for example, the remaining sites might be occupied by oxygens from other carboxylate groups, phosphate groups, hydroxyls, amides, or even bound water molecules (Byler & Farrell, 1989). In human lactoferrin, the iron is coordinated by 2 Tyr, 1 Asp and 1 His \{Tyr 92(435) and 192(528), Asp 60(395) and His 253(597) in the N(C)-lobes\}. Two oxygens from the bidentate carbonate ion complete a distorted octahedral coordination round the iron (Baker et al., 1991). In a typical calcium binding protein, α-lactalbumin, Ca\(^{2+}\) is coordinated by all oxygens, three from the carboxylate groups of aspartyl residues (Asp 82, 87and 88), two carbonyl oxygen atoms (from Asp 79 and 84) and two from water molecules (Stuart et al., 1986). Because FTIR is unable to provide this kind of information, details about Ca\(^{2+}\) binding sites of lactoferrin remain unknown. Inter-molecular coordination, in which octahedral coordination around Ca\(^{2+}\) are occupied by the carboxylate ligands from different lactoferrin molecules, can be reconciled with the fact that lactoferrin forms tetrmers in the presence of Ca\(^{2+}\) (Bennett et al., 1981).

Relative movement of domains is a common feature of multi-domain proteins and is frequently crucial for biological function (Bennett & Huber, 1984). The two-lobe, four-domain structure of lactoferrin allows considerable scope for flexibility, which could include relative movement of the N- and C-lobes, or of the two domains which comprise each lobe (Baker et al., 1991), and this appears to be a vital factor in its function (Baker & Lindley, 1992). The nature and extent of this change has been graphically demonstrated by the X-ray structure analysis of apo-human lactoferrin
(Anderson et al., 1990), with an indication that it undergoes the structural changes from "open" to "close", associated with binding and release of iron. This structure suggests a plausible model for the mechanism of iron-binding, in which the metal first binds to domain II (N2 or C2), with the cleft open, after which the domain closes over the metal to complete its coordination (Baker & Lindley, 1992).

The opening of the cleft (the open form of apoprotein) exposes three basic side chains, previously buried within it; Arg121, Arg210 and Lys301 (Anderson et al., 1990). When the domains close in human lactoferrin as iron is bound, a salt bridge, Lys301...Glu216 is formed near the iron site in the hinge region (Baker & Lindley, 1992). This salt bridge derives from a local conformational change in human lactoferrin where a flip of the peptide 302-303 allows Lys301 to approach Glu216 (Baker & Lindley, 1992). The same interaction also happened with Glu211 of N2, which forms a salt bridge with Arg89 of N1 (Anderson et al., 1990). Existing only in diferric human lactoferrin (Baker & Lindley, 1992), the salt bridge between Lys301 (or Arg89) and Glu216 (or Glu211) seems to play a role like a "hitch" between two domains (N1 and N2) when the interdomain cleft is closed as iron is bound (Anderson et al., 1990; Baker & Lindley, 1992).

As we know that BLf is highly similar to HLf in having a bilobal tertiary molecule and characteristic iron-binding (Spik et al., 1993), domain movement with a salt bridge linking and breaking is probably a common feature of lactoferrins of different origins. The details of HLf tertiary structure mentioned above can be used to discuss the possible effects of Ca$^{2+}$ on the domain movement of native lactoferrins. If the salt bridges Lys301...Glu216 and Arg89...Glu211 are broken due to Ca$^{2+}$ binding
to Glu216 and Glu 211, the "closed" form of lactoferrin may be greatly distorted, or normal conformational changes of lactoferrin with "open" and "closed" structures may be seriously affected, so that its functions are lost. A hypothetic model is proposed in this study as shown in Fig. 4-14 which gives a detailed description. This could be a likely explanation for why antimicrobial ability of lactoferrin can be blocked in the presence of Ca$^{2+}$ (Ellison et al., 1990; Ellison & Giehl, 1991).

The secondary structures such as α-helix, β-sheet, and turns of apo-lactoferrin and of apo-lactoferrin with different ions were probed by FTIR. The original spectra (Fig. 4-6), after Fourier self-deconvolution (Fig. 4-15), were subject to band fitting, and the percentages of secondary structures have been thus estimated by adding the areas of all bands assigned to each of these structures and expressing the sum as a percentage of the total amide I' band area from 1700-1620 cm$^{-1}$. Fig. 4-15 has given original (dots) and deconvolution (solid lines) spectra of all samples studied in which many overlapped bands undetected in the original spectra have been shown, and the changes of positions and shapes associated with the presence of metal ions are obvious. Fig. 4-16 has shown deconvoluted amide I' (solid line) and synthetic (dots) band contours together with the individual component bands.

Tables 4-3 and 4-4 give positions and percentage areas of the individual components, as well as their assignments. From these raw data given in Table 4-5, some main points can be concluded as follows.

(a) α-Helix and β-sheet of apo-Lf pellets are 47.2% and 34.8%, respectively, compared with that of human lactoferrin by X-ray crystallography, e.g 41% α-Helix and 24% β-sheet (Anderson et al., 1989).
(b) \(\alpha\)-Helix and \(\beta\)-sheet of apo-Lf in D\(_2\)O solution, on the other hand, are 38.0% and 32.9%, separately, lower than that of pellet form, compared with that of human apolactoferrin in H\(_2\)O solution by FTIR, 43% \(\alpha\)-helix and 27% \(\beta\)-sheet (Haden et al., 1994).

(c) Addition of Ca\(^{2+}\) to apolactoferrin has resulted in decrease of \(\alpha\)-helix and increase of \(\beta\)-sheet. Additions of K\(^+\) and Na\(^+\) to apolactoferrin have caused a decrease in both \(\alpha\)-helix and \(\beta\)-sheet. Except for \(\alpha\)-helix reduction by 19% in K-Lf, all other changes are small (decrease of 1-5%). Bovine lactoferrin in the presence of Ca\(^{2+}\), shows the greatest changes of secondary structures among the samples of the solid state, namely an increase of 23% in \(\beta\)-sheet and decrease of 27% in \(\alpha\)-helix. Furthermore, bovine lactoferrin with Ca\(^{2+}\) present in the liquid state has lost nearly half of its \(\alpha\)-helix (46%).

(d) When bovine lactoferrin binds iron, there was a slight decrease in \(\beta\)-sheet and increase in \(\alpha\)-helix (6%) content. However, because the changes are so small, this result can be interpreted as no change in the secondary structure of Lf on binding iron, which is consistent with the result reported by Haden et al. (1994).

Such conformational rearrangements of lactoferrin on interaction with metal ions, particularly Ca\(^{2+}\), will surely influence the functional behaviour of the protein. Lactoferrin is an \(\alpha/\beta\) protein, in the notation of Levitt & Chothia (Levitt & Chothia, 1976), and the \(\alpha\)-helices generally run antiparallel to the \(\beta\)-sheet (Anderson et al., 1989). Most of them thus run away from the interdomain cleft, with their N termini contributing to the walls of the cleft (Baker & Lindley, 1992). This normal structure
could be changed when hydrogen bonds in the α-helix are presumably broken by Ca²⁺ ion which causes a loss of helix, resulting in the reverse of some biological functions.

Both hLf and bLf can be bound to the surfaces of animal and microbial cells via lactoferrin receptors (Spik et al., 1993; Pettersson & Tommassen, 1994). These studies have indicated that the lactoferrin region (HLf or BLf) involved in the interactions with the lymphocyte receptor is the sequence of residues 4-52 on the N-lobe of domain I (Spik et al., 1993). The conformational comparisons of two peptides originating from BLf and HLf have indicated that the two peptides consist of an α-β-α-β structure and are identical according to superposition (Spik et al., 1993).

The peptides derived from gastric pepsin cleavage of both human and bovine lactoferrin were found to have the bactericidal activity at least eightfold greater than that of the original lactoferrins (Tomita et al., 1991; Bellamy et al., 1992a). A physiologically diverse range of Gram-positive and Gram-negative bacteria is susceptible to inhibition and inactivation by this peptide. The bactericidal domains of human lactoferrin (named lactoferricin H) and bovine lactoferrin (named lactoferricin B) have been isolated and sequenced (Bellamy et al., 1992b). This peptide was found to be derived from the N-terminal region of the molecule. It consists mainly of a loop of 18 amino acid residues formed by a disulfide bond between cysteine residues 20 and 37 of human lactoferrin, or 19 and 36 of bovine lactoferrin (Bellamy et al., 1992b). This bactericidal peptide is the part of receptor-binding region of lactoferrin (amino acid residues 4-52) and would possess α-β structure according to X-ray analysis of lactoferrin. By studying interaction of lactoferricin B with microbial cells, ¹⁴C-labeled peptide has been found to be bound rapidly to the surface of E. coli. and B. subtilis
(Bellamy et al., 1993a) and to C. albicans (Bellamy et al., 1993b). The rates of binding were consistent with the rates of killing induced by the peptide.

In brief, the sequence (residues 4-52) of BLf and HLf, designated as the "binding sequence", is located on the apex of the N-domain "hump" and possesses α/β secondary structure. This sequence is responsible for binding animal and microbial cells, and such binding is critical for bactericidal and fungicidal effects of lactoferricin or lactoferrin. However, the bactericidal ability of lactoferrin will be blocked in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) (Ellison et al., 1990; Ellison & Giehl, 1991) whereas antibacterial effectiveness of the lactoferricin B is inhibited by Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\) or Mg\(^{2+}\) ions (Bellamy et al., 1992a), or simply by increasing ionic strength (Jones et al., 1994).

In this study, Ca\(^{2+}\) has been found to bind with the carboxylic side-chains and greatly distort the secondary structure of lactoferrin. On the other hand, the results show that Na\(^{+}\) and K\(^{+}\) do not specifically bind with the carboxylic side-chains but slightly change the secondary structure of lactoferrin. The results obtained in this study can be used to explain the blockage of antimicrobial ability by Ca\(^{2+}\) or ionic strength, if one accepts the possibility that a certain α/β structure of the "binding sequence" is necessary for lactoferrin or lactoferricin to interact with the microbial cell.

If Ca\(^{2+}\) interacts with the carboxylates of Glu and Asp residues in the "binding sequence" (residues 4-52) and the carbonyl oxygen of a peptide linkage nearby, a certain α/β structure of that sequence might be distorted, in consequence lactoferrin would not bind or bind only inefficiently, to the surface of bacteria, thus reversing antibacterial activity. Na\(^{+}\) and K\(^{+}\) have been found not to interact with carboxylic side-
chains of lactoferrin, thus these ions would influence secondary structures in unspecific ways to a much lesser extent. This means that these ions might not distort the α/β structure of the “binding sequence” of lactoferrin, resulting in little effect on antibacterial activity. As lactoferrin is digested by pepsin to produce the active peptide with only 18 residues, the α/β structure of the small peptide might be easily influenced and changed by ionic strength, thereby making the bactericidal peptide inactive.

4.4 CONCLUSION

The conclusions that can be drawn from the above studies on the interaction of bovine lactoferrin with some metal ions, particularly calcium include:

(1) Ligand binding of lactoferrin with calcium has been detected by spectrophotometry and fluorimetry. Bovine lactoferrin (BLf) in the presence of Ca$^{2+}$ has shown small but clear changes of both ultraviolet absorption and fluorescence as compared with apo-lactoferrin (apo-Lf). This results indicates an attachment of Ca$^{2+}$ to Lf. A low association constant for Ca$^{2+}$ binding Lf, $K_{dss} = 1.98 \times 10^4$ M$^{-1}$ was obtained with a "direct calcium titration" using the absorption difference (ΔA) at 280 nm. The value of about n = 5 for the number of multiple-binding sites of calcium was also calculated from the direct titration curve.

(2) Interactions of side chains of Lf, particularly carboxylic groups, with metal ions such as Ca$^{2+}$, Na$^+$, K$^+$ and Fe$^{3+}$, have been investigated by FTIR. The results have suggested that Ca$^{2+}$ and Fe$^{3+}$ do interact with carboxylate groups of Lf as shown by the fact that a band of apo-Lf at 1400.8 cm$^{-1}$, arising from the O-C-O stretching
vibrations of COO\(^-\), is apparently lost when Ca\(^{2+}\) is present or the band is shifted to give a weak shoulder near 1410 cm\(^{-1}\) with iron present. On the other hand, the spectrum of lactoferrins with Na\(^+\) and K\(^+\) do not show any change in the band near 1400 cm\(^{-1}\) as compared with apo-Lf's, suggesting that disappearance of this band in the sample of Lf with Ca\(^{2+}\) is not due to the effect of ionic strength but to binding between side-chains of Lf and calcium. In addition, there is good agreement in the changes of the characteristic band near 1400 cm\(^{-1}\) for samples prepared in KBr pellet and in D\(_2\)O solution.

(3) In the region of 1500-1350 cm\(^{-1}\), the original spectra of apo-Lf and those with K\(^+\) or Na\(^+\) have shown two peaks, one near 1450 cm\(^{-1}\), and the other near 1400 cm\(^{-1}\). When Ca\(^{2+}\) and Fe\(^{3+}\) are bound to Lf, the band at 1400 cm\(^{-1}\) disappeared or became a shoulder while intensities of the band near 1450 cm\(^{-1}\) are increased. It is thus proposed in this study that the band near 1450 cm\(^{-1}\) be assigned to COOH and COOCa(Fe), compared with the band at 1400 cm\(^{-1}\) which refers to COO\(^-\).

(4) The difference spectrum of Lf + Ca\(^{2+}\) minus apo-Lf has been used to estimate the coordination structure of carboxylates of Lf with Ca\(^{2+}\), based on the rules derived from the studies of acetate-Ca\(^{2+}\) and carboxylates-Ca\(^{2+}\) in caseins. These data suggests that the binding of Ca-carboxylate in bovine lactoferrin is either chelation [II] or bridging [III].

(5) The sensitive amide I' band (1700-1620 cm\(^{-1}\)) has been used for analysis of secondary structures of lactoferrin as well as detection of possible changes due to interactions of metal ions. The data have indicated that in the presence Ca\(^{2+}\), the amounts of the secondary structures of lactoferrin such as \(\alpha\)-helix, \(\beta\)-sheet, turns etc.,
have undergone a great alteration whereas K⁺ and Na⁺ have just caused a slight change. On the contrary, Fe³⁺ gives almost no changes in α-helix and β-sheet of lactoferrin. Ca²⁺ exerted the greatest influence on α-helix and β-sheet changes in lactoferrin with a decrease of 27-46% and a increase of 19-23%, respectively, implying that Ca²⁺ binds with carboxylates of Glu or Asp residues in an α-helix or β-sheet and also coordinates with carbonyl groups of peptides links nearby.

(6) These results have been used to explain the experimental observations that Ca²⁺ ions reduce or reverse the antimicrobial capability of lactoferrin. The effects supposedly involved include, Ca²⁺ breaking down a salt bridge, e.g. Lys301...Glu216, that is important for the "closed" form of diferric lactoferrin (Baker & Lindley, 1992), and the altering of a certain α/β structure in the “binding sequence” of lactoferrin (Spik et al., 1993) on the supposition that a certain α/β structure of the “binding sequence” is necessary for lactoferrin or lactoferricin to attach to the microbial cell and kill it.

*νₘₚₚ(CO2): Asymmetrical stretching vibration of carboxylate, cm⁻¹.
*νₘₚₚ(CO2): Symmetrical stretching vibration of carboxylate, cm⁻¹.
*Δ: νₘₚₚ(CO2) - νₘₚₚ(CO2)
4.5 REFERENCES


APPENDIX 4-1: TABLES
IN CHAPTER 4
TABLE 4-1
Comparisons of Calcium-Binding $K_{\text{ass}}$ and Sites between Lactoferrin and Other Proteins

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$K_{\text{ass}}$(M$^{-1}$)</th>
<th>$pK_{\text{ass}}$</th>
<th>$Ca^{2+}$ Sites No.($n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Lf@</td>
<td>$1.28 \times 10^4$</td>
<td>4.3</td>
<td>5</td>
</tr>
<tr>
<td>ConA#</td>
<td>$3.3 \times 10^3$</td>
<td>3.5</td>
<td>1</td>
</tr>
<tr>
<td>CaM#</td>
<td>$10^5, 10^6$</td>
<td>5, 6</td>
<td>4</td>
</tr>
<tr>
<td>TnC#</td>
<td>$10^5, 10^7$</td>
<td>5, 7</td>
<td>4</td>
</tr>
<tr>
<td>$\alpha$-La#</td>
<td>$10^6 - 10^9$</td>
<td>6-9</td>
<td>1</td>
</tr>
<tr>
<td>Human Lf*</td>
<td>$10^{20}$</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

ConA: ConcanavalinA; $\alpha$-La: $\alpha$-lactalbumin; CaM: Calmodulin; TnC: Troponin; $pK_{\text{ass}}$: log$K_{\text{ass}}$.

@ Calcium-binding of bovine lactoferrin.

# Calcium-binding of various proteins (Strynadka & James, 1989).


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TABLE 4-2
The Spectral Assignments for the Carboxylates in Bovine Lactoferrin$^a$

<table>
<thead>
<tr>
<th>Types of Carboxylates</th>
<th>Band Assignments</th>
<th>Absorbance Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Approx. cm$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>R-COO$^-$</td>
<td>1400$^c$, 1401$^b$</td>
<td>0.4175$^c$, 0.0345$^b$</td>
</tr>
<tr>
<td>R-COOH</td>
<td>1449$^c$, 1452$^b$</td>
<td>0.4276$^c$, 0.0473$^b$</td>
</tr>
<tr>
<td>R-COOFe</td>
<td>1440$^c$</td>
<td>1.2448$^c$</td>
</tr>
<tr>
<td>R-COOCa</td>
<td>1449$^c$, 1456$^b$</td>
<td>0.7068$^c$, 0.1014$^b$</td>
</tr>
</tbody>
</table>

$^a$ Data collected from Figs.4-6 and 4-10. The band assignments based on the original spectra.

$^b$ Aqueous solutions.

$^c$ KBr pellets.
### TABLE 4-3
Positions and Fractional Areas of the Amide I Bands of Bovine Lactoferrins in KBr Pellets$^a$

|            | apo-LF: |       |       |       |       |       |       |       |       |       | Ca-LF: |       |       |       |       |       |       |       |       | K-LF: |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |�
| position (cm$^{-1}$) | 1627 | 1635 | 1639 | 1647 | 1658 | 1666 | 1670 | 1677 | 1682 | 1689 | 1693 | 1627 | 1636 | 1640 | 1647 | 1655 | 1658 | 1667-70 | 1677 | 1682 | 1689 | 1693 | 1628 | 1635 | 1639 | 1647 | 1658 | 1666 | 1670 | 1678 | 1682 | 1689 | 1693 | 1627 | 1635 | 1638 | 1646 | 1658 | 1666 | 1670 | 1677 | 1682 | 1689 | 1693 | 1627 | 1638 | 1649 | 1658 | 1669 | 1677 | 1682 | 1692 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |�
| area (%) | 17.1 | 0.9  | 6.7  | 10.9 | 47.2 | 0.2  | 0.6  | 10.1 | 0.7  | 4.5  | 1.1  | 24.8 | 0.9  | 7.5  | 8.1  | 0.4  | 33.9 | 5.4  | 9.7  | 2.6  | 4.2  | 2.3  | 15.0 | 1.5  | 9.5  | 13.2 | 38.1 | 0.3  | 6.0  | 7.8  | 1.5  | 5.8  | 1.4  | 15.8 | 0.6  | 9.4  | 9.9  | 44.8 | 0.8  | 0.9  | 8.5  | 2.0  | 4.0  | 3.3  | 17.7 | 10.0 | 3.1  | 50.0 | 1.9  | 5.0  | 2.6  | 9.7  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |�
| assign.*  | β    | β    | β    | U    | α    | T    | T    | β    | T    | T    |       | β    | β    | U    | α    | α    | T    | β    | T    | T    |       | β    | β    | U    | α    | T    | T    | β    | T    | T    |       | β    | β    | U    | α    | T    | β    | T    | T    |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |�

$a$: Assignments for amide I band components (1700-1620 cm$^{-1}$) based on Byler & Susi, 1988.

$β$: β-Structure, α: α-Helix, U: Unordered (aperiodic), T: Turns, assign.*: assignment.

Samples, apo-LF: apolactoferrin, Ca-LF: apolactoferrin + CaCl$_2$, K-LF: apolactoferrin + KCl, Na-LF: apolactoferrin + NaCl, Fe-LF: apolactoferrin + FeCl$_3$.
Table 4-4

Positions and Fractional Areas of the Amide I Bands of Bovine Lactoferrins in D$_2$O Solution$^a$

<table>
<thead>
<tr>
<th></th>
<th>apo-lactoferrin</th>
<th></th>
<th></th>
<th>apolactoferrin + Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>band position</td>
<td>band area</td>
<td>assignments$^a$</td>
<td>band position</td>
</tr>
<tr>
<td></td>
<td>(cm$^{-1}$)</td>
<td>( % )</td>
<td></td>
<td>(cm$^{-1}$)</td>
</tr>
<tr>
<td>1625</td>
<td>8.5</td>
<td>β-Structure</td>
<td></td>
<td>1625</td>
</tr>
<tr>
<td>1632</td>
<td>12.3</td>
<td>β-Structure</td>
<td></td>
<td>1632</td>
</tr>
<tr>
<td>1638</td>
<td>10.2</td>
<td>β-Structure</td>
<td></td>
<td>1638</td>
</tr>
<tr>
<td>1643</td>
<td>9.8</td>
<td>Unordered</td>
<td></td>
<td>1642</td>
</tr>
<tr>
<td>1650</td>
<td>9.4</td>
<td>α-Helix</td>
<td></td>
<td>1650</td>
</tr>
<tr>
<td>1656</td>
<td>28.6</td>
<td>α-Helix</td>
<td></td>
<td>1655</td>
</tr>
<tr>
<td>1660-5</td>
<td>9.4</td>
<td>Turns</td>
<td></td>
<td>1659</td>
</tr>
<tr>
<td>1671</td>
<td>4.4</td>
<td>Turns</td>
<td></td>
<td>1665</td>
</tr>
<tr>
<td>1676</td>
<td>1.9</td>
<td>β-Structure</td>
<td></td>
<td>1672</td>
</tr>
<tr>
<td>1681</td>
<td>3.3</td>
<td>Turns</td>
<td></td>
<td>1677</td>
</tr>
<tr>
<td>1586</td>
<td>1.9</td>
<td>Turns</td>
<td></td>
<td>1681</td>
</tr>
<tr>
<td>1692-7</td>
<td>1.0</td>
<td>Turns</td>
<td></td>
<td>1686</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1692</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1697</td>
</tr>
</tbody>
</table>

$^a$ Assignments for amide I' band components (1700-1620 cm$^{-1}$) based on Byler & Susi, 1988.
Table 4-5
Secondary Structure Analysis of Bovine Lactoferrins

<table>
<thead>
<tr>
<th>Structures (%)</th>
<th>KBr Pellets</th>
<th>D2O Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>apo-Lf</td>
<td>Ca-Lf</td>
</tr>
<tr>
<td>α-Helix</td>
<td>47.2</td>
<td>34.3</td>
</tr>
<tr>
<td>α-change*</td>
<td>-27</td>
<td>-19</td>
</tr>
<tr>
<td>β-Sheet</td>
<td>34.8</td>
<td>42.9</td>
</tr>
<tr>
<td>β-change*</td>
<td>+23</td>
<td>-3</td>
</tr>
<tr>
<td>Unordered</td>
<td>10.9</td>
<td>8.1</td>
</tr>
<tr>
<td>U-change*</td>
<td>-26</td>
<td>+21</td>
</tr>
<tr>
<td>Turns</td>
<td>7.1</td>
<td>14.5</td>
</tr>
<tr>
<td>T-change*</td>
<td>+104</td>
<td>+111</td>
</tr>
</tbody>
</table>

\[
\text{Cation -Lf} = \text{apo-Lf} \\
\text{* change (%) } = \frac{\text{Cation -Lf}}{\text{apo-Lf}} \times 100
\]

Cation includes Ca, Na, K, or Fe.
APPENDIX 4-2: FIGURES IN CHAPTER 4
Fig. 4-1 Absorption difference spectra between apo-Lf and apo-Lf + Ca$^{2+}$. Apo-Lf was in the reference beam. The buffers were 0.05 M acetate, pH 4.5 (I), and 0.05 M Tris, pH 7.5 (II). The protein concentration was 18.7 mM. The curve b is the base-line traces (apo-Lf vs. apo-Lf), and the curve a is the trace of apo-Lf + 0.14 mM Ca$^{2+}$.

Fig. 4-2 Comparison of fluorescence spectra between apo-Lf and apo-Lf + Ca$^{2+}$. The curve A is apo-Lf + 0.14 mM Ca$^{2+}$, and the curve B is apo-Lf. The excitation wavelength was 292 nm. The measurements were made in 0.05 M Tris buffer, pH 7.5, 25°C, in a protein concentration of 31.2 mM.
Fig. 4-3 Titration Curves of Lactoferrin with Ca2+ at Different pHs.
The conditions of titration are shown in the text. Series 1: titration in Tris buffer, pH 7.5; Series 2: titration in Acetate buffer, pH 4.5.

Fig. 4-4 Scatchard Plot of Titration Curve at pH 7.5.
Series 1: Experimental data, Series 2: Data of regression analysis.
Fig. 4-5 Original FTIR spectra of lactoferrins with the solid sampling in the region of 4000-400 cm⁻¹. (A) apo-Lf + Fe³⁺; (B) apo-Lf + Ca²⁺; (C) apo-Lf + Na⁺; (D) apo-Lf; (E) apo-Lf + K⁺.

Fig. 4-6 Original FTIR spectra of lactoferrins with the solid sampling in the region of 1800-1100 cm⁻¹. (F) apo-Lf + Fe³⁺; (G) apo-Lf + Ca²⁺; (H) apo-Lf + Na⁺; (I) apo-Lf; (J) apo-Lf + K⁺.
Fig. 4-7 Second derivative spectra of Na\textsuperscript{+}-Lf (a), apo-Lf (b), and K\textsuperscript{+}-Lf (c) with solid sampling. These spectra were calculated from original spectra shown in Fig.4-5.

Fig. 4-8 Second derivative spectra of Ca\textsuperscript{2+}-Lf (-----) and apo-Lf (-----) with solid sampling. These spectra were calculated from original spectra shown in Fig.4-5. The data of wavenumbers and absorbance indicate the sample of Ca\textsuperscript{2+}-Lf.
Fig. 4-9 Second derivative spectra of Fe$^{3+}$-Lf (---) and apo-Lf (------) in solid sampling. These spectra were calculated from original spectra shown in Fig. 4-5. The data of wavenumbers and absorbance indicate the sample of Fe$^{3+}$-Lf.

Fig. 4-10 Original spectra of Ca$^{2+}$-Lf (d) and apo-Lf (e) in D$_2$O solution.
Fig. 4-11 Second derivative spectra of Ca$^{2+}$-Lf (——) and apo-Lf (—-—) in D$_2$O solution. These spectra were calculated from original spectra shown in Fig. 4-10. The data of wavenumbers and absorbance indicate the sample of Ca$^{2+}$-Lf.

Fig. 4-12 The bands of the tyrosin residue for lactoferrins. Arrow-indicated peaks are assigned to the C-C stretching vibration of the tyrosin aromatic ring in different forms of the lactoferrins. (a) Ca$^{2+}$-Lf in solid sampling, (b) apo-Lf in solid sampling, (c) Ca$^{2+}$-Lf in D$_2$O solution, (d) Fe$^{3+}$-Lf in solid sampling. These peaks were selected from second derivative spectra shown in Fig. 4-8, 4-9, 4-11.
Fig. 4-13 Difference spectrum calculated from the two original spectra of apo-Lf and Ca\(^{2+}\)-Lf in Fig. 4-5. The spectrum of apo-Lf (Fig. 4-5 D) is subtracted from the spectrum of Ca\(^{2+}\)-Lf (Fig. 4-5 B). The ordinate represents the computed difference in the absorbance intensities at each data point.

---

Fig. 4-14 Schematic representation of the structural changes associated with binding and release by native lactoferrin with and without Ca\(^{2+}\) using human lactoferrin as model

Model A (Absence of Ca\(^{2+}\)): A lobe (N or C) of lactoferrin undergoes the structural change from "open" (left) to "close" (right) by binding iron, during which a salt bridge Glu216...Lys301 is formed [13,14,16,35].

Model B (Presence of Ca\(^{2+}\)): Supposing Ca\(^{2+}\) binds with Glu216, lactoferrin fails to undergo the structural change from "open" (left) to "close" (right)
Fig. 4-16 Amide I' and synthetic band contours with the best-fitted individual component bands for different forms of the lactoferrin. Deconvolved spectrum contour and individual components are solid lines (—). Synthetic spectrum contour obtained by summation of the components is a dotted line (---).
(A): Ca^{2+}-Lf, (B): Fe^{3+}-Lf, (C) K^{+}-Lf, (D): Na^{+}-Lf, (E): apo-Lf in solid sampling; (F): apo-Lf, and (G)(H): duplicates of Ca^{2+}-Lf in D_{2}O solution.
Chapter 5

Separation of Immunoglobulin and Glycomacropeptide from Whey

ABSTRACT

Concentration of immunoglobulins (Igs) in whey by selectively removing major whey proteins such as α-lactalbumin (La), β-lactoglobulin (Lg) and bovine serum albumin (BSA) has been studied using the anion exchanger IRA93 and Amicon YM100 membrane.

IRA93 can selectively adsorb Lg and La around pH 7.0 without seriously removing Igs in whey. Quantity of resin and frequency of treatment with IRA93 have also been tested for their effects on recovery of La and Lg. Different cation exchange resins were tried to adsorb Igs selectively from whey at pH 5.0 but they were unsuccessful.

In this study, ultrafiltration (UF) was another technique used for the concentration of Igs in whey. The effects of pH, NaCl, and diafiltration (DF) on recovery of La, Lg and BSA have also been studied. The studies have suggested in agreement with some other reports (Thomas et al., 1992) that fractionation of whey proteins is greatly influenced by alterations in the charge carried by the whey proteins. NaCl added to whey has a negative effect on the recovery of La, Lg and BSA though the extent of the effect depends heavily on pH. This study also suggests that more proteins of lower MW can be removed by an limited DF, but repeated diafiltration is of little help. Separation efficiency (SE) of IRA93 and the membrane technique has been compared; and higher SE was obtained from IRA93.

A process with IRA93 and 100 kDa membrane treatment has been used to produce commercial-like products containing Igs from acid and colostral whey. The Igs content of the product determined by the ELISA technique, is 43.3% for acid whey and 93% for colostral whey, respectively.

IRA93 has also been found to selectively adsorb glycomacropeptide (GMP) from Cheddar cheese whey at pH 4.7.
5.1 INTRODUCTION

Many studies have been done on the separation of immunoglobulins (Igs) and glycomacropeptide (GMP) from cheese whey. Igs can be separated from low-molecular weight proteins such as α-lactalbumin (La) and β-lactoglobulin (Lg) by one or two-stage ultrafiltration (Kothe et al., 1987; Scoot & Lucas, 1989), and immunoglobulinG (IgG) can also be isolated by copper chelating chromatography (Al-Mashikhi et al., 1988), gel filtration chromatography (Al-Mashikhi & Nakai, 1987), and microfiltration affinity purification (Chen & Wang, 1991). On the other hand, GMP can be isolated by ultrafiltration (UF) and Q-Sepharose ion-exchange chromatography (Tanimoto et al., 1992).

Proteins are commonly characterized by their iso-electric points (pI), the pH at which they have zero net charge. This net charge has commonly been used to predict the behavior of proteins on ion exchange resins based on the assumptions that proteins will not be retained at their pI, that they will be retained by anion exchangers at pH’s above their pI or by cation exchange resins below their pI (Durance, 1987). In addition to pI, proteins also have different molecular weights (MW) which are usually used to predict the behavior of proteins in molecular-sieve separations such as membrane technology and gel filtration chromatography.

The pIs and MWs of whey proteins are well known (Eigel et al., 1984; Kinsella & Whitehead 1989). La, Lg and bovine serum albumin (BSA) are weakly acidic proteins with a pI of 4.8, 5.3 and 5.1, respectively whereas Igs have higher pIs. IgG₃ has a pI of 5.5-6.8 and IgG₂ a pI of 7.7-8.3. La and Lg are smaller proteins with a MW of 14.2 and 18.6 kilo-Dalton (kDa), respectively. Igs, on the other hand, have a
molecular weight in the range of 150 - 200 kDa. In addition, GMP is a highly acidic and hydrophilic peptide with a MW of 7 kDa (Kinsella & Whitehead 1989). On the basis of these properties, a method can be developed with the aim of enriching the target proteins by removal of the impurities like La and Lg or isolating the target proteins such as Igs and GMP directly.

In this study, a polystyrene matrix anion exchanger and UF were used for separation of Igs from La and Lg; the anion exchanger was also used for selective adsorption of GMP from Cheddar cheese whey.

5.2 MATERIALS AND METHODS

5.2.1 Whey Samples

Cheddar cheese whey from Norco Co. Ltd., Lismore, and cottage cheese whey from ACF, were sampled on the day of cheese manufacture and colostrum was obtained from the farm of the University of Western Sydney, Hawkesbury. All samples were transported to the laboratory at 5°C without pasteurization. Fat was removed by centrifugation at 37°C. Acid and Colostral wheys were made from raw skimmed milk and defatted colostrum, respectively, by precipitation of the casein by 1 N HCl at pH 4.6. The casein was removed by filtration. Casein precipitation and de-fatting were carried out on the day of receipt at the laboratory. Wheys were packed in plastic bottles with one litre capacity and frozen at -20°C. Frozen whey was thawed at 2-4°C overnight before use.
For the isolation of Igs, whey samples were pretreated by adjusted to pH 6.5 and above by addition of 6 N NaOH, then centrifuged in the Sorvall RC-5B (Du Pont Instruments) at 5000 x g and 30 min to remove the precipitate.

5.2.2 Ion Exchange Resin and Batch Adsorption

The ion exchange resins used in this study are listed in Table 1. All resins were treated with 0.5 M HCl and NaOH and washed extensively with deionised water prior to use. The resin was then equilibrated with 0.05 M acetate or phosphate buffer at appropriate pH value. The standardised conditions of batch adsorption used by Dionysius et al. (1991) have been adopted in this study.

For the batch operation, free water was removed from the resin under vacuum using a Buchner flask and sintered glass filter funnel. Pretreated colostral and acid wheys (for isolation of Igs) or defatted Cheddar whey (for isolation of GMP) mentioned above was adjusted to the required pH with 6 N HCl or NaOH, added to the moist resin in an Erlenmeyer flask, gently shaken on a shaking platform or in a beaker, and agitated with a stirrer at room temperature for 30 min. The whey-resin mixture was then transferred to a sintered-glass filter funnel, and the spent whey (the whey after treatment by the resin) recovered under vacuum. The resin was then washed with 2 volumes water, followed by elution of bound proteins with 1 volume of 1 M NaCl in the equilibrium buffer. Effects of the parameters such as resin quantity, resin type and treating frequency on protein adsorption, were also studied.

For the studies on some resins, such as C-150S, SP-Sephadex and SE-Productiv™, a Poly-Prep column (Bio-Rad) with dimensions 0.8 x 4 cm was used. Four ml resin in the Poly-Prep column were equilibrated with 0.05 M acetate buffer at
pH 5.0 and 15 ml sample at pH 5 was applied. The resin was washed with 0.05 M acetate buffer at pH 5.0. The sample was eluted with 0.18 M Tris at pH 8.5.

5.2.3 Separation of Igs by Ultrafiltration (UF)

Amicon thin-channel system with membrane YM100 (100 kDa cut-off) was used for separation of Igs from low MW proteins in pretreated whey. The whey was pumped through the membrane by using a peristaltic pump to give the retentate and permeate. Diafiltration (DF) was achieved by adding water to the retentate and ultrafiltering again.

The Rejection ($R_r$) and Permeability ($R_p$) of the membrane is expressed by (Renner and El-Salam, 1991):

$$R_r = 1 - \frac{C_p}{C_r} \quad (5-1)$$
$$R_p = 1 - R_r \quad (5-2)$$

$C_p$ is the solute concentration in the solution passing through the membrane (permeate)

$C_r$ is the solute concentration upstream of the membrane (retentate)

5.2.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Quantitative Measurement by Scanning

SDS-polyacrylamide gels, PSDS-130, MSDS-1020 and GSS-1020 (Gradiopore Ltd., Sydney), and gel electrophoresis apparatus (Gradiopore Ltd.), were used for this purpose. Electrophoresis was performed in the buffer system (lower and upper) recommended by Gradiopore Ltd.; Tris 9.2 mM, Tricine 20 mM, SDS 0.1%, pH 7.5. 10 µl mixture of 50 µl sample, 50 µl 4% SDS, 50 µl 8 M urea, 10 µl 2-mercaptoethanol (1.1168 g/ml, Sigma) and 10 µl 0.1 % bromophenol blue in 50 % glycerine solution, were loaded onto the gel and electrophoresed at a constant 150 volts until bromophenol blue moved out of the gel. Gels were then agitated for 1 hr in the
fixing solution composed of 50% ethanol, 40% water and 10% glacial acetic acid, then stained for 30 min in the staining solution containing 0.5% Coomassie blue, 0.5% amido schwarz 10B in the fixing solution. Acetic acid 3% solution was used as destaining agent. Bovine IgG (ICN Biomedicals) was used as a standard.

The quantitative measurement of whey proteins on Coomassie blue staining gel was based on Hillier’s method (Hillier, 1976). A photograph of stained gel was scanned by a Shimadzu Dual-Wavelength TCL Scanner CS-910 and plotted using the integrator, LDC/Milton Roy C1-10. For Coomassie blue stained gels, optical transmission method was used at a wavelength of 600 nm. The important parameters in this analysis include slit width (W) and height (H) of light beam, binary attentuation factor (BAF, values from 0 to 9), and chart speed (CS, 0-125 mm/min). For comparison, the same parameters were always used.

The content of Igs to total whey protein (represented by all proteins on SDS-PAGE gel), i.e. purity of Igs, was determined from % of Ig’s peak area (PA) to total peak areas of all proteins. The peak area of Igs (PA_{IgS}) was estimated by the method of Kaneko et al. (1985), where the heavy chain peak area was multiplied by a coefficient of 1.4 because the determination of light chain peak area was difficult due to overlapping with other minor protein components. However, a coefficient of 1.5 was used in this study from an analysis of the IgG standard purchased from ICN Biomedicals.

5.2.5 Evaluation of Separation Efficiency

The concept of separation efficiency (SE), which was proposed by Kaneko et al. (1985), has been used to evaluate the efficiency of Igs enrichment by ion exchange and UF. Separation efficiency (SE) is expressed here as:
SE = Igs ratio in spent whey or retentate / Igs ratio in original whey \hspace{1cm} (5-3)

Igs ratio is defined as the ratio of Igs to the sum of BSA, Lg and La calculated from the peak area of Igs (PA_{Igs}), BSA (PA_{BSA}), Lg (PA_{Lg}) and La (PA_{La}) on the scanning patterns:

\[ \text{Igs ratio} = \frac{\text{PA}_{Igs}}{\text{PA}_{BSA} + \text{PA}_{Lg} + \text{PA}_{La}} \] \hspace{1cm} (5-4)

5.2.6 Evaluation of Protein Removal (%)

Protein removal (PR) is determined by comparison in the scanner peak height of specific protein between spent and pretreated whey. The equation is expressed as:

\[ \text{PR} \hspace{0.1cm} \text{%} = (1 - \frac{\text{Peak height of the protein in spent whey}}{\text{Peak height of the protein in pretreated whey}}) \times 100 \] \hspace{1cm} (5-5)

In order to compute appropriately in the case of IRA 93 treating frequency (Table 5-3, Fig. 5-3 a, b), the Protein Reduction (PD) is introduced here as:

\[ \text{PD} \hspace{0.1cm} \text{%} = (1 - \frac{\text{Peak height of the protein in following spent whey}}{\text{Peak height of the protein in previous spent whey}}) \times 100 \] \hspace{1cm} (5-6)

5.2.7 Dialysis

Whey was transferred to Spectrapor membrane tubing with MW cutoff 6000-8000, then dialysed against 20 volumes of deionised water (renewed every 12 hr) at 4°C for 48 hr.

5.2.8 Analysis of Amino Acid and Protein Sequence of GMP Fraction

A fraction supposedly containing GMP was treated with 12% trichloroacetic acid (TCA) to precipitate the contaminants. The supernatant was dialysed in Spectrapor membrane tubing with MW cutoff 3500 to remove TCA. The dialysed supernatant solution was then lyophilized for sequencing.
The amino acids in the sample were analysed by AAA — Waters Picotag System by Deakin Research Ltd.

The N-terminus of the sample was sequenced to residue 38 on an Applied Biosystems 470A Protein Sequencer by Deakin Research Ltd.

5.2.9 Enzyme-linked Immunosorbent Assay (ELISA)

The antibody-sandwich protocol for ELISA (Coligan, 1991) has been used to quantify the concentration of Igs in Igs-containing powder. The method for the quantification of rat anti mouse IgA, practised at the UNSW Department of Microbiology and Immunology, Australia, was used in this study.

Stock Reagent Solutions:

Coating Buffer:  
0.01 M Carbonate Buffer pH 9.6  
\[ \text{Na}_2\text{CO}_3 \] 1.59 g  
\[ \text{NaHCO}_3 \] 2.93 g  
\( \text{H}_2\text{O} \) up to 1 litre

**TW / PBS**  
0.1% Tween 20 PBS (0.12 M NaCl) pH 7.2  
\( \text{Na}_2\text{HPO}_4 \) 21.4 g  
\( \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} \) 7.8 g  
\( \text{NaCl} \) 140.2 g  
Tween 20 20 ml  
\( \text{H}_2\text{O} \) up to 20 litre

**0.01 M Citrate Acetate Buffer pH 6.0**  
Solution A:  
\( \text{Na acetate} \) 8.2g  
\( \text{H}_2\text{O} \) up to 1 litre

Solution B:  
\( \text{Citric acid.H}_2\text{O} \) 2.1 g  
\( \text{H}_2\text{O} \) up to 100 ml

Add small quantities of solution B to Solution A until pH reaches 6 (about 10 ml B to 1 liter A). Store at 4°C.

**TMB Solution**  
3′-3′, 5′-5′ tetramethylbenzidine (TMB) (Sigma)  
TMB 175 mg  
Methanol 50 ml
Reagents for Analysis, for 2 plates:

Plate: Dynatech Immulon™

Coating Buffer: 0.01 M carbonate buffer pH 9.6

Coating Antibody: Anti-cow Igs (Rabbit Igs, DAKOPATTS), 1mg/ml

Coating Solution: Anti-cow Igs 10 μl/ml in coating buffer

Blocking buffer: 1% chicken ovalbumin in coating buffer

Diluting Buffer: 1% chicken ovalbumin in TW / PBS

Conjugate: Peroxidase labelled rabbit anti-cow Igs, DAKOPATTS

Conjugate Solution: 0.05% conjugate / 0.1% NMS in diluting buffer

- 10 μl conjugate
- 20 μl NMS
- 20 ml diluting buffer
  - prepare just before use

Substrate Solution: 25.8 ml 0.01 M citrate acetate buffer pH 6

- 0.8 ml TMB solution
- 4 ml 30% H₂O₂
  - prepare just before use

Stop Solution: 2 M H₂SO₄

Procedures for Analysis

The plate of Dynatech Immulon™ for ELISA is illustrated as follows:

(Plate for ELISA)

<table>
<thead>
<tr>
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</tr>
</tbody>
</table>
where wells A1 to H1 (black wells) are designated for blanks; wells {A2, B2 (duplicate wells)} to {C12, D12} are designated for serial dilution of the standard bovine IgG (ICN Biomedicals), and wells {E2, F2} to {G12, H12} for serial dilution of the samples.

1). Coating of Plate with Antibody:

Add 100 μl coating solution to each well of plates

Incubate at 37°C for 3 hr

Wash plates 3 times with TW/PBS and once with water

2). Blocking:

Add 100 μl blocking buffer to each well of plates

Incubate at 37°C for 3 hr

Wash plates 3 times with TW/PBS and once with water

3). Addition of Standard, Sample Dilutions:

Add 100 μl diluting buffer to wells of the blank

Add 100 μl diluting buffer to wells for serial dilution of the standard and samples

Add 200 μl of the standard and sample to wells {A2, B2} and {E2, F2}, respectively and carry out doubling serial dilution (100 μl)

Incubate at 37°C for 1 hr

Wash plates 3 times with TW/PBS and once with water

4). Addition of Conjugate:

Add 100 μl conjugate solution to each well of plates

Incubate 37°C for 1 hr

Wash plates 3 times with TW/PBS and once with water
5). Color Development:

Add 100 μl substrate solution to each well
Stop reaction by adding 50 μl stop solution

6). Absorbance and Data:

Read absorbance at 450 nm in Model 3550 Microplate Reader
Report by the Microplate Manager / Macintosh Software (Bio-Rad)

5.2.10 Other Analyses

Ultraviolet and visible spectrophotometry were performed on a Perkin Elmer lambda 2 UV/Vis spectrophotometer equipped with software PECSS version 3.2. Total whey protein (TWP) was determined by a modified micro-Biuret method (Sleigh, 1982) or absorbance at 280 nm.

5.3 RESULTS AND DISCUSSION

5.3.1 Enrichment of Igs by Selective Removal of La, Lg and BSA

The ion exchange resin, Amberlite IRA 93, was chosen for this purpose. Fig. 5-1 shows that the eluates of IRA 93 contain mainly La and Lg (Lanes 1 and 5). This result indicated that IRA 93 selectively binds La and Lg at pH 7.5 but not Igs. All immunoglobulins (Butler, 1969) appear to be either monomers or polymers of a four-chain molecule consisting of two light polypeptide chains (L-chains: 20,000 MW) and two heavy chains (H-chains) with MWs varying from 50,000 to 70,000 for different Igs classes. These H- and L-chains are linked by disulphide-bonds. When 2-mercaptoethanol is used in SDS-PAGE, the disulphide-bond is cleaved and the H- and L-chains move independently.
Table 5-2 and Fig. 5-2 show the effect of resin quantity on the removal of La and Lg. It would be expected that the increase of in protein removal keeps pace with the increase of resin quantity. When 100 g IRA 93 was added to 100 ml whey, 75.3% of La and 73.0% of Lg were removed from the whey. Multistage treatment using 20 g of resin to 100 ml whey (Table 5-3 and Fig. 5-3 a, b) with three extractions can remove 88.1% of La and 90.7% of Lg from whey. Efficiency of multistage treatment is evidently higher than that of single treatment. However, a negative side of multistage treatment is that Igs is also removed from the whey. There are three reasons for this loss. First, IgG₂, accounting for 25% of total IgG (Bjorck, 1991), can be retarded by Sephadex DEAE A-50 at pH 6.5 (Hladik & Kas, 1973) because it is a more basic protein (pI 7.7-8.3). Secondly, repeated treatment by the anion exchanger will cause the pH value of whey to go up, resulting in more Igs being adsorbed on the resin. Thirdly, the \textit{net charge concept}, namely “zero” retention at or very near the pI on ion exchange resin is inaccurate for native proteins that deviate significantly from predicted retention behaviour by binding to ion exchange resins at their pI (Durance, 1987; Kopaciewicz et al., 1983). In addition, IRA 93 appears to have more difficulty removing BSA, although BSA is also a weakly acidic protein (pI 5.1).

\textbf{5.3.2 Selective Adsorption of Igs by Cation Exchange}

Theoretically, the environment of pH 5.0 allows most Igs to be positively charged so that they can be selectively bound to cation exchange resins while La, Lg and BSA are excluded because they have a net zero charge at pH 5.0. However, the experimental results did not agree with this theoretical assumption based on the \textit{net charge concept}. No cation exchanger used in this study selectively adsorbed Igs. Except for resin C-150 (Fig. 5-4 C, D), all other resins give completely “empty”
eluates (Lane 1 or Lane d in Fig. 5-4). C-150 can weakly bind a little Lg and a protein that could be casein.

According to the net charge concept, a protein should be immediately retained on the cation exchange resin at the pH below its pI or on the anion exchange resin at the pH above its pI, and the protein retention should parallel net charge (Kopaciewicz et al., 1983). However, the poor correlation between the protein retention and the net charge concept has been found because of charge asymmetry (Kopaciewicz et al., 1983). For example (Kopaciewicz et al., 1983), β-glucosidase (almonds) has a pI of 7.3, but it can hardly bind to the Mono S cation-exchange resin at pH 4.5 (ca. three pH units below its pI). The other reason is that the protein retention on ion exchange resin is influenced substantially by the counter-ion (Kopaciewicz et al., 1983). Cations in whey such as K\(^+\), Na\(^+\) and Ca\(^{2+}\) have been found to reduce the protein retention on cation exchanger about 50% (i.e. 0.5 of relative retention).

5.3.3 Selective Concentration of Igs by 100 kDa UF Membrane

It has been reported that enrichment of Igs in whey can be made by removal of low MW proteins through UF technology (Kothe et al., 1987; Scoot & Lucas, 1989; Kirihara & Yamamoto; 1990; Thomas et al., 1992). Thomas et al. (1992) have examined the effect of pH on the separation of low MW whey proteins to enrich Igs by using charged, formed-in-place membranes. They have found that as the pH increased the rejection of the proteins was reduced. In contrast, the rejection of the whey proteins increased with the increase of pH when Amicon YM 100 membrane with slightly negative charge was used in this study (Fig. 5-5 A). Since the isoelectric points (pI) of the major whey proteins (La, Lg and BSA) range from 4.8 to 5.3, the increase of pH resulted in an increase in total negative charge. So, it is not difficult to understand that
the whey proteins under higher pH condition will be highly rejected by negatively charged membranes. All results including previous and present work, have indicated that better separation of ionic compounds by UF membranes depends on both charge and size characteristics. Using Amicon YM 100 membrane to treat whey without addition of NaCl, permeability of Lg and La are 80% and 98% at pH 4.3 as compared with those of 34% and 43% at pH 9.0 (Fig. 5-5 A, B, C), respectively.

Fig. 5-5 B,C,D have shown the effect of NaCl on the permeability of the whey proteins such as La, Lg and BSA. As the concentration of salt increased at pH 4.3 the permeability of La and Lg in whey dramatically decreased (rejection increased). La showed the biggest drop in permeability, from 98% without salt to 20% with 5% salt. Maillliart & Ribadeau-Dumas (1988) have reported that whey proteins excepting Lg can be salted out by NaCl at low pH. This finding can be used to explain the result of this study, where the drop in permeability on adding NaCl to whey at pH 4.3 may be due to the aggregation of the whey proteins. By adding NaCl to whey at higher pHs (pH 7.0 and 9.0), the permeability does not show a great fluctuation, which may be because the permeability has not been seriously affected by salt at the higher pH. This result conflicts with a report (Renner & El-Salam, 1991) in which additions of NaCl increased the permeability of La and Lg when ultrafiltering milk. The patterns from scanning SDS-PAGE gels for Fig.5-5 are shown in Fig.5-6a, b.

Diafiltration (DF) by adding water to retentate then ultrafiltering is a technology widely used in UF processing to remove more impurities. The relationship between rejection and DF has been studied (Fig. 5-7 and 5-8). First diafiltration (DF-1) for the UF retentate gives a significant drop in rejections of La, Lg and BSA against membrane (improvement of the permeability). From the second diafiltration (DF-2),
rejection starts to increase until goes up sharply for the third diafiltration (DF-3). The result has implied that in UF technology an effort to achieve more purification by DF is limited. Comparison between ion exchange and UF in separation efficiency (SE) for selectively removing impurities is shown in Fig. 5-9. SE rose exponentially on multistage treatment by the ion exchanger and SE increased arithmetically on multistage treatment with UF (UF + DF).

Table 5-4 gives the result of commercial-like products containing Igs enriched from HCl-casein and colostral whey by the process combined with IRA93 resin and 100 Da UF membrane treatment. The powder produced from HCl-casein whey by this process contains 43.3% Igs based on total solids (TS) while the product made from Cheddar cheese whey by single UF membrane technology (Thomas et al., 1990) had 20% Igs based on total whey protein (TWP). In the case of colostral whey, this process has produced a product containing 93% Igs compared with 63% Igs by single UF treatment (Kirihara & Yamamotou, 1990). Igs contents in acid and colostral whey were measured in this study as 6.6 and 49.5% of TWP, respectively, which agrees with the range reported in the literature (Eigel et al., 1984; Kinsella & Whitehead, 1989; Kirihara & Yamamotou, 1990; Bjorek, 1991; Oyenini & Hunter, 1978). The very high concentration factor of Igs (72-fold) was obtained from acid whey treated by this process. Fig. 5-10 and 5-11 have shown the reports of Bio-Rad Microplate Manager 2.1 and details of the calculation for ELISA analysis of Igs contents in the powders.

5.3.4 Selective Adsorption of GMP by IRA93

GMP is a very acidic peptide and it moved towards the anode (positive electrode) on paper electrophoresis at pH 4.7. However, most other whey proteins are positively charged or have a net zero charge at pH 4.7 (Eigel et al., 1984; Kinsella &
Whitehead., 1989). Therefore a process could be set up on the assumption that GMP is able to be selectively removed from Cheddar cheese whey by an anion exchange resin at pH 4.7. Fig.5-13 gives the pattern of SDS-PAGE for the eluate of IRA 93 adsorption (Lane A) which shows about four bands. It is difficult to determine which one is GMP from SDS-PAGE without a standard. According to results of SDS-PAGE of Morr & Seo (1988), GMP was found at the same position as β-lactoglobulin. In addition, other studies have also indicated (Kawasaki et al., 1993) that the apparent MW of GMP was pH-dependent. Although the theoretical MW of GMP was about 7 kDa, the retention time of gel filtration chromatography indicated the MW distributed from 20 to 50 kDa at pH 7.0. At pH 3.5, on the contrary, five major peaks were detected at MW ranging from 10 to 30 kDa. The mechanism for the pH-dependent MW changes in GMP is unclear. One possibility is that GMP associates itself to form oligomers at neutral pH through non-covalent interactions, and the oligomers partially dissociate at acidic pH. In addition, since the GMP is quite heterogeneous, mainly due to variable amounts of carbohydrate attached to the original k-casein, it might be the carbohydrate that is causing the anomalous elution behaviour on gel filtration.

So, the second band from the bottom is probably GMP. The eluate of IRA93 adsorption was further treated with 12% TCA to precipitate impurity proteins. As only GMP is soluble in 12% TCA solution (Armstrong et al., 1976), if present it should remain in the supernatant after TCA treatment. The absorbance at 270 nm in the UV/Vis spectrophotometric scan (Fig.5-13) indicates that the dialysed supernatant treated with 12% TCA still contains contaminants as peptides because the GMP has no aromatic acids. This supernatant was lyophilized and results of the amino acids and sequence analysis are shown in Table 5-5 and Fig. 5-15, respectively. The amino acid
content (residues/mol) of the TCA soluble fraction is very close to theoretical (Table 5-5). Only 38 residues of the fragment in the fraction have been sequenced as shown in Fig. 5-15 (II), the N-terminal residue is Met, and the amino acid sequence from M¹ to V³⁸ is identical to that of GMP (Fig. 5-15, I). The experimental results have confirmed that the eluate from IRA 93 at pH 4.7 contains GMP.

So far, a few commercially viable technologies have been reported for isolating GMP from whey or the casein mixture. A patented process (Kawasaki and Dosako, 1991) was based on removal of impure proteins by ion exchange chromatography then purification by UF membrane at pH below 4. By comparison, this GMP-separating method is selective adsorption with one step treatment of ion exchange resin though further study on separation is still needed. When this preliminary work is improved by more studies, the process with the selective adsorption of GMP from whey by IRA 93 will show an advantage over the patented one.

5.4 CONCLUSION

Concentration of immunoglobulins (Igs) in whey by selectively removing major whey proteins such as α-lactalbumin (La), β-lactoglobulin (Lg) and bovine serum albumin (BSA) has been studied by using the anion exchanger IRA 93 and Amicon YM100 membrane.

IRA93 has been found to selectively adsorb Lg and La at pH’s around 7.0 under the condition that the resin also bound 20% of Igs in whey. Quantity of resin and frequency of treatment with IRA93 have been tested for their effects on the recovery of
La and Lg, indicating that increasing frequency of treatment give more efficiency than increasing resin quantity but not effective for recovery of Igs in the whey.

Although selective adsorption of Igs on the cation exchanger at ca. pH 5.0 can be predicted by the net charge concept, the reality shows a different story in which almost no protein in whey at ca. pH 5.0 has been bound to the tested cation exchange resins such as Amberlite IRC 150, Purolite C-150S, SP-Sephadex G-25 and SE-Productiv™.

In this study, ultrafiltration (UF) was another technique used for the concentration of Igs in whey. The effects of pH, NaCl, and diafiltration (DF) on recovery of La, Lg and BSA have also been studied. In general, UF is regarded as a method of separation based on size. However, this study has shown in agreement with some other studies (Thomas et al., 1992) that fractionation of whey proteins is greatly influenced by alterations in the charge carried by the whey proteins. Because most membranes carry some charges, the membrane separation technique is based on both size and charge in a broad sense. Since the Amicon YM 100 used in this study is slightly negatively charged, it has been found that rejection of La, Lg, and BSA was significantly reduced with increasing positive charge on the protein (i.e reducing pH values). On the other hand, NaCl added to whey totally has a negative effect on the recovery of La, Lg and BSA though the extent of the effect depends heavily on pH. This study also suggests that the permeability can be improved (i.e. rejection reduced) by one DF, but repeated treatment with diafiltration is not much help for removal of more contaminating proteins (i.e. rejection dramatically increased). Separation efficiency (SE) of IRA 93 and the membrane technique has been compared; higher SE was obtained by IRA 93.
A process using IRA 93 and 100 kDa membrane treatment has been used to produce commercial-like products containing Igs from acid and colostral whey. The Ig content of the product, determined by the ELISA technique, is 43.3% for acid whey and 93% for colostral whey, respectively.

IRA 93 was also used for adsorption of glycomacropeptide (GMP) from Cheddar cheese whey. This was possible because GMP is negatively charged at pH 4.7. Since the GMP contained in the eluate of IRA93 has been suggested by SDS-PAGE and confirmed by amino acid and sequence analysis, it can be concluded that GMP is selectively bound to IRA 93 at pH 4.7.

A process for isolation of Igs and GMP compiled from the results of this study is proposed in Fig. 5-15. To achieve a commercial process, further work is required as follows:

(1) Optimization of the process for selective adsorption of GMP by the resin IRA 93.

(2) Further investigation for selective adsorption of Igs from whey by cation exchange resins (shown as optional process in Fig. 5-15) since this process is the best option in overall consideration.

This chapter is a step on the path to implementing the _Total Whey Utilization Scheme_ in Fig.1-6, where the protein concentrate of pretreated whey by UF will be further separated to the fractions of major whey proteins, Igs and GMP by the processes in Fig.5-15.
5.5 REFERENCES


APPENDIX 5
TABLES AND FIGURES
IN CHAPTER 5
Table 5-1 Lists of Ion Exchange Resins

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Ion exchange</th>
<th>Matrix Structure</th>
<th>Functional Groups</th>
<th>Manufacturers</th>
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<tr>
<td>Amberlite IRA 93</td>
<td>Weak anion</td>
<td>Styrene -DVB</td>
<td>$- \text{N}-(\text{R})_2$</td>
<td>Rohm &amp; Haas</td>
</tr>
<tr>
<td>Amberlite IRC 150</td>
<td>Weak cation</td>
<td>Methacrylic -DVB</td>
<td>Carboxyl</td>
<td>Rohm &amp; Haas</td>
</tr>
<tr>
<td>Purolite C-150S</td>
<td>Strong cation</td>
<td>Styrene -DVB</td>
<td>Sulpho</td>
<td>The Purolite Company</td>
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<tr>
<td>SP-Sephadex, G-25</td>
<td>Strong cation</td>
<td>Dextran</td>
<td>Sulphophosphoryl</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>SE-Productiv™</td>
<td>Strong cation</td>
<td>Cellulose</td>
<td>Sulpho-ethyl</td>
<td>BPS Separations</td>
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</tbody>
</table>

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**Fig.5-1 SDS-PAGE — Selective Adsorption of La and Lg by IRA93.** Lane 1: eluate-2, Lane 2: lactoferrin standard, Lane 3: spent whey-2, Lane 4: IgG standard, Lane 5: eluate-1, Lane 6: IgG standard, Lane 7: spent whey-1, Lane 8: defatted Cheddar whey.

200 ml defatted (sample 1) and pretreated (sample 2) Cheddar wheys were each mixed with 100 g wet resin (IRA 93) at pH 7.5, and kept for 1 hr at room temperature. Filtration, washing, and elution were then used to obtain spent wheys and eluates which were run by SDS-PAGE.
Table 5-2
Effect of Amount of Resin on the Removal of α-Lactalbumin and β-Lactoglobulin

<table>
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<th>Resin IRA 93 (g / 100 ml whey)</th>
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<th>100</th>
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<tr>
<td>Protein</td>
<td>Protein Removal (PR%)</td>
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<td>α-Lactalbumin</td>
<td>51.5</td>
<td>54.9</td>
<td>75.3</td>
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<tr>
<td>β-Lactoglobulin</td>
<td>20.0</td>
<td>44.4</td>
<td>73.0</td>
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To three 100 ml samples of pretreated whey were added 20, 50 and 100 g wet resin at pH 7.2. The pretreated and spent wheys were run by SDS-PAGE, all sample loads being 2 μl. Amounts of La and Lg were determined by scanning the gels of SDS-PAGE (Fig. 5-2).

Fig. 5-2 Profiles of scanned SDS-PAGE gels for the data in Table 5-2. (A) and (B) were on different gel from (C), (D) and (E). The parameters used for all samples include BAF = 7, chart speed = 20. Slit width of (A) and (B) was W = 0.5 and slit height H = 10 mm and that of (C), (D) and (E) was W = 0.25 and H = 7 mm.

(A): pretreated whey. (B): spent whey treated with 20 g resin /100 ml. (C) pretreated whey. (D): spent whey treated with 50 g resin /100 ml. (E): spent whey treated with 100 g resin /100 ml.
Table 5-3
Effect of Frequency of Treatment by IRA93 on Removal of Whey Proteins

<table>
<thead>
<tr>
<th>Protein Removal, (PR%)</th>
<th>Treatment of IRA93</th>
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<tr>
<td></td>
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<td>TWP</td>
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<tr>
<td>La</td>
<td>51.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lg</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BSA</td>
<td>0</td>
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<tr>
<td>IgG*</td>
<td>20</td>
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Pretreated acid whey was continuously treated with IRA93 4 times at pH 7.5. The quantity of resin was kept at 20 g/100 ml for each treatment. The samples of spent whey underwent electrophoresis (Fig. 5-3a). All sample loads were 2 μl.

<sup>a</sup> PR% of La and Lg was measured directly from Fig. 5-3b (A); <sup>b</sup>,<sup>c</sup>,<sup>d</sup> PR% of La and Lg was determined indirectly from PD% with Fig. 5-3b (A); <sup>b</sup> PR% = (100% - 81%) x 37.4% + 81%; <sup>c</sup> PR% = [(100% - 88.1%) x 58.8%] + 88.1%; <sup>d</sup> PR% = [(100% - 90.7%) x 70.5%] + 90.7%.

BSA and IgG were measured from Fig.5-3b (B). TWP was determined by a spectrophotometer at 280 nm.

<sup>*</sup> IgG was calculated as heavy chains of IgG in SDS-PAGE.

---

**Fig. 5-3a** Photographs of SDS-PAGE gels for the Experiments in Table 5-3. Lane 1: pretreated acid whey, Lane 2: IgG standard, 1mg/ml, Lane 3: 1st spent whey, Lane 4: 2nd spent whey, Lane 5: 3rd spent whey, Lane 6: 4th spent whey, Lane 7: MW Marker.
Fig. 5.3b Profiles of scanned SDS-PAGE gels for Fig. 5.3a. (A): parameters were set for the determination of La and Lg, including BAF = 7, chart speed = 20, slit width (W) = 0.5 mm and slit height (H) = 10 mm. (B): parameters were set for the determination of BSA and IgG-H, including BAF = 5, chart speed = 20, W = 0.15 mm and H = 6.14 mm. (1) and (I): pretreated acid whey, (2) and (II): 1st spent whey, (3) and (III): 2nd spent whey, (4) and (IV): 3rd spent whey, (5) and (V): 4th spent whey.
Fig. 5-4 SDS-PAGE Gels — Selective Adsorption of Igs by Various Cation Exchange Resins. A: 6SE-Productiv™, *CH; B: 4SE-Productiv™, +CO; C: 4Purolite C-150, *CH; D: 4Purolite C-150, +CO; E: 4SP-Sephadex G-25, *CH; F: 4SP-Sephadex G-25, +CO; G: IRC 150, *CH.

+ Trade name of resins. * CH = Cheddar cheese whey, CO = Cottage cheese whey.
Lane 1: The eluate; Lane 2: Wash fraction; Lane 3: Spent whey; Lane a: IgG standard; Lane b: Pretreated whey; Lane c: Spent whey; d: The eluate.
2-mercaptoethanol was not used in SDS-PAGE for the samples A, B, C, D, E, and F. So, IgG only shows one band on the gels.
Fig. 5-5 Effects of pH and NaCl on Rejection and Permeability of Whey Proteins by UF

Pretreated acid whey was adjusted to pH 4.3, 7.0 and 9.0, then solid NaCl was added to give 1%, 3% and 5%. These samples (including the control without adding NaCl) were concentrated 6 times by a 100 kDa membrane. The retentates and permeates of ultrafiltration were measured by a spectrometer at 280 nm for TWP, and were run in SDS-PAGE. La, Lg and BSA were determined by scanning the SDS-PAGE gels.

TWP: Total Whey Protein; La: α-Lactalbumin; Lg: β-Lactoglobulin; BSA: Bovine Serum Albumin.

Permeability (%) of TWP = (A_{280nm} of permeate / A_{280nm} of retentate) x 100. Permeability (%) of La, Lg and BSA = (peak height of the protein in permeate / peak height of the protein in retentate) x 100

Rejection (%) = 100% - Permeability (%)
Fig. 5-6a Profiles of scanning SDS-PAGE gels for the experiments in Fig. 5-5.
(1) Ret of 4.3* and 0%\(^{\circ}\); (2) Per of 4.3* and 0%\(^{\circ}\); (3) Ret of 7.0* and 0%\(^{\circ}\); (4) Per of 7.0* and 0%\(^{\circ}\); (5) Ret of 9.0* and 0%\(^{\circ}\); (6) Per of 9.0* and 0%\(^{\circ}\); (7) Ret of 4.3* and 1%\(^{\circ}\); (8) Per of 4.3* and 1%\(^{\circ}\); (9) Ret of 7.0* and 1%\(^{\circ}\); (10) Per of 7.0* and 1%\(^{\circ}\); (11) Ret of 9.0* and 1%\(^{\circ}\); (12) Per of 9.0* and 1%\(^{\circ}\);
Ret: Retentate; Per: Permeate; * pH value; \(^{\circ}\) NaCl concentration.
Parameters of the scanner and integrator: BAF = 7, slit width = 3.4 mm and slit height = 8.4 mm, chart speed = 20.
Fig. 5-6b Profiles of scanned SDS-PAGE gels for the experiments in Fig. 5-5.
(13) Ret of 4.3* and 3%³; (14) Per of 4.3* and 3%³; (15) Ret of 7.0* and 3%³; (16) Per of 7.0* and 3%³; (17) Ret of 9.0* and 3%³; (18) Per of 9.0* and 3%³; (19) Ret of 4.3* and 5%⁴; (20) Per of 4.3* and 5%⁴; (21) Ret of 7.0* and 5%⁴; (22) Per of 7.0* and 5%⁴; (23) Ret of 9.0* and 5%⁴; (24) Per of 9.0* and 5%⁴;
Ret: Retentate; Per: Permeate; * pH value; º NaCl concentration.
Parameters of the scanner and integrator: BAF= 7, slit width = 3.4 mm and slit height = 8.4 mm, chart speed = 20.
Fig. 5-7 Rejection ($R_\text{r}$) of TWP, La, Lg and BSA by Ultrafiltration (UF) and Diafiltration (DF) in a 100 kDa Membrane. 1600 ml pretreated acid whey was ultrafiltered to give 8-fold concentrations. Then the retentate was made up to the original volume (1600 ml) by addition of water, and diafiltered to give another 8 fold concentration. Diafiltration was carried out 3 times. The retentates and permeates of ultrafiltration were measured at 280 nm for TWP, and were run in SDS-PAGE. La, Lg and BSA were determined by scanning the SDS-PAGE gels.

UF: ultrafiltration, DF-1: 1st diafiltration, DF-2: 2nd diafiltration, DF-3: 3rd diafiltration

Rejection (%) of La, Lg and BSA = \left\{ \frac{1 \text{ - (peak height of the protein in permeate / peak height of the protein in retentate)}}{ } \right\} \times 100
Fig. 5-8 Profiles of scanned gels for the experiments in Fig. 5-7. (a) Ret of UF, (b) Per of UF; (c) Ret of DF-1, (d) Per of DF-1; (e) Ret of DF-2, (f) Per of DF-2; (g) Ret of DF-3, (h) Per of DF-3.

Ret: retentate; Per: permeate.
Parameters of the scanner and integrator: BAF = 7, slit width = 4.0 mm and slit height = 9.0 mm, chart speed = 30.
Fig. 5-9 Separation Efficiency for Enrichment of Igs by Ion Exchange and UF Treatment. S-1: spent whey of 1st resin treatment or retentate of UF; S-2: spent whey of 2nd resin treatment or retentate of 1st DF; S-3: spent whey of 3rd resin treatment or retentate of 2nd DF; S-4: spent whey of 4th resin treatment or retentate of 3rd DF.

Table 5-4
Igs-containing Powder Made by IRA 93 and UF Treatment

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<tr>
<th></th>
<th>AW(^a)</th>
<th>Ig Powder (I)(^b)</th>
<th>CW(^a)</th>
<th>Ig Powder (II)(^b)</th>
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<td>Igs Purity(^*) (%)</td>
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<td>N/A</td>
<td>49.5</td>
<td>N/A</td>
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<td>Igs Content(^@) (g/100g TS(^b))</td>
<td>0.6(^c)</td>
<td>43.3(^d)</td>
<td>34.7(^e)</td>
<td>93.0(^d)</td>
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<td>Concentration Factor (Fold)(^#)</td>
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\(^a\) AW = Acid Whey; CW = Colostral Whey; TS = Total Solids.

\(^b\) Ig Powder (I) from acid whey; Ig Powder (II) from collostral whey.

\(^*\) Purity of Igs is determined by % of peak area (PA) of Igs relative to all proteins in SDS-PAGE gel.

\(^@\) Igs content is determined by both the peak area of Igs in SDS-PAGE gel and ELISA. The peak area of Igs (PA(Ig)) was estimated by multiplying the heavy chain peak area by a coefficient 1.5.

\(^c\) calculated from the peak area of Igs in SDS-PAGE gel. \(^d\) determined by ELISA.

\(^#\) concentration factor = Igs content of powder / Igs content of whey.

500 ml of pretreated acid whey and 200 ml acid collostral whey were added to 20 g/100 ml wet IRA93, and the reaction was carried out at pH 7.0-8.0 for 30 min. Spent whey was obtained by filtering the resin, and was ultrafiltrated by 10-fold concentrations then diafiltrated once in 100 kDa membrane. Ig-rich powders were obtained by freeze-drying.
(I): Raw Data

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(II) Curve Fit

Plot: Log-Logit; Abs. = (A - D) / (1 + (Conc. / C)^B) + D
Coeff: A = 5.2576 - 02  B = 1.2163  C = 3.1796 - 02  D = 1.9863
Std. Err: 0.293 absorb units

(III) Calculation Table

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<th>Std.Dev.</th>
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<th>Conc.</th>
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Standards: 1 (A2,B2) | 1.990 | 0.017 | 0.85 | 10.000 |
| 2 (A3,B3) | 1.967 | 0.008 | 0.39 | 5.000 |
| 3 (A4,B4) | 1.982 | 0.025 | 1.29 | 2.500 |
| 4 (A5,B5) | 1.989 | 0.015 | 0.75 | 1.250 |
| 5 (A6,B6) | 1.963 | 0.004 | 0.16 | 0.6250 |
| 6 (A7,B7) | 1.811 | 0.047 | 2.28 | 0.3125 |
| 7 (A8,B8) | 1.891 | 0.058 | 3.43 | 0.1526 |
| 8 (A9,B9) | 1.473 | 0.031 | 2.11 | 7.8125E-02 |
| 9 (A10,B10) | 1.085 | 0.060 | 5.54 | 3.9662E-02 |
| 10 (A11,B11) | 0.660 | 0.015 | 2.25 | 1.9531E-02 |
| 11 (A12,B12) | 0.423 | 0.120 | 28.28 | 9.7656E-03 |
| 12 (C3,D3) | 0.093 | 0.064 | 68.34 | 4.8828E-03 |
| 13 (C4,D4) |  |  |  |  |
| 14 (C5,D5) |  |  |  |  |

Fig. 5-10 Igs content in Ig-rich powder of acid whey — Report of Bio-Rad Microplate Reader 2.1.
(I) standard IgG of 1 mg/ml, was diluted from 1:100 (well {A2,B2}) to 1:2097,1520,0 (well {G12,H12}) except well {C2,D2}; sample of 4.0 mg/ml, was diluted from 1:100 (well {E2,F2}) to 1:2097,1520,0 (well {G12,H12}) except well {G2,H2}. (II) Absorbances from about 0.66 to 1.47 are linear with concentrations, and used for calculation. (III) the sample dilutions labelled by * fit in with the absorbance ranges for calculation. Igs concentration, µg/ml, is obtained by multiplying Conc. of the labelled sample by its dilution then averaging these samples.
(I): Raw Data

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(II) Curve Fit

Plot: Log-logistic: Abs. = (A - D) / (1 + Conc. / C)^-B + D
Conc.: A = 0.5, B = 5.52E-03, C = 1.3212, D = 1.660E-02
Std. Err.: 0.0381

(III) Calculation Table

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<td>0.30534</td>
</tr>
<tr>
<td>5</td>
<td>[E6,F6]</td>
<td>2.312</td>
<td>0.083</td>
<td>3.56</td>
<td>0.1331</td>
</tr>
<tr>
<td>6</td>
<td>[E7,F7]</td>
<td>2.310</td>
<td>0.046</td>
<td>1.99</td>
<td>0.1379</td>
</tr>
<tr>
<td>7</td>
<td>[E8,F8]</td>
<td>2.242</td>
<td>0.006</td>
<td>0.28</td>
<td>9.7958E-02</td>
</tr>
<tr>
<td>8</td>
<td>[E9,F9]</td>
<td>2.184</td>
<td>0.012</td>
<td>0.55</td>
<td>8.0703E-02</td>
</tr>
<tr>
<td>R</td>
<td>[E10,F10]</td>
<td>2.021</td>
<td>0.018</td>
<td>0.87</td>
<td>5.2954E-02</td>
</tr>
<tr>
<td>10</td>
<td>[E11,F11]</td>
<td>1.676</td>
<td>0.020</td>
<td>1.18</td>
<td>3.0473E-02</td>
</tr>
<tr>
<td>11</td>
<td>[E12,F12]</td>
<td>1.232</td>
<td>0.050</td>
<td>4.08</td>
<td>5.7365E-02</td>
</tr>
<tr>
<td>12</td>
<td>[E13,F13]</td>
<td>2.046</td>
<td>0.057</td>
<td>2.71</td>
<td>8.2324E-03</td>
</tr>
<tr>
<td>13</td>
<td>[G4,H4]</td>
<td>0.298</td>
<td>0.025</td>
<td>11.85</td>
<td>4.2764E-03</td>
</tr>
<tr>
<td>14</td>
<td>[G5,H5]</td>
<td>0.074</td>
<td>0.016</td>
<td>20.95</td>
<td>1.8701E-03</td>
</tr>
<tr>
<td>15</td>
<td>[G6,H6]</td>
<td></td>
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<td></td>
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<td>16</td>
<td>[G7,H7]</td>
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<td>17</td>
<td>[G8,H8]</td>
<td></td>
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<td></td>
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<tr>
<td>18</td>
<td>[G9,H9]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>[G10,H10]</td>
<td>0.048</td>
<td>0.000</td>
<td>0.00</td>
<td>1.5701E-03</td>
</tr>
<tr>
<td>20</td>
<td>[G11,H11]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>[G12,H12]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Standards:|                |        |          |      |       |
| 1        | [A2,B2]        | 2.531  | 0.091    | 3.60 | 10.000|
| 2        | [A3,B3]        | 2.369  | 0.127    | 5.34 | 5.0000|
| 3        | [A4,B4]        | 2.521  | 0.000    | 0.00 | 2.5000|
| 4        | [A5,B5]        | 2.399  | 0.046    | 2.00 | 1.2500|
| 5        | [A6,B6]        | 2.388  | 0.030    | 1.27 | 0.6250|
| 6        | [A7,B7]        | 2.402  | 0.005    | 0.20 | 0.1250|
| 7        | [A8,B8]        | 2.366  | 0.012    | 0.50 | 0.15625|
| 8        | [A9,B9]        | 2.157  | 0.020    | 0.95 | 7.1825E-02|
| 9        | [A10,B10]      | 1.846  | 0.017    | 0.92 | 3.9062E-02|
| 10       | [A11,B11]      | 1.302  | 0.043    | 3.31 | 1.9521E-02|
| 11       | [A12,B12]      | 0.801  | 0.105    | 13.06| 9.7656E-03|
| 12       | [C3,D3]        | 0.349  | 0.030    | 8.72 | 4.6828E-03|
| 13       | [C4,D4]        | 0.123  | 0.025    | 20.65| 2.4414E-03|

Fig. 5-11 Igs content in Ig-rich powder from colostrum whey — Report of Bio-Rad Microplate Manager 2.1. (I) standard IgG of 1 mg/ml, was diluted from 1:100 (well A2,B2) to 1:2097,1520,0 (well G12,H12) except well (C2,D2); sample of 1.9 mg/ml, was diluted from 1:100 (well E2,F2) to 1:2097,1520,0 (well G12,H12) except well (G2,H2). (II) Absorptions from about 0.6 to 1.8 are linear with concentrations, and used for calculation. (III) The sample dilutions labelled by * fit in with the absorbance ranges for calculation. Igs concentration, μg/ml, is obtained by multiplying Conc. of the labelled sample by its dilution then averaging these samples.
Fig. 5-12 Photograph of SDS-PAGE of the eluate from IRA 93. Lane A: eluate, Lane B: defatted Cheddar whey.
1 litre of defatted Cheddar whey at pH 4.7 was treated with 100 gram wet IRA 93 with stirring, then filtered through a sintered glass filter funnel where unbound impurities on the resin were washed off by 2 volumes of water. The fraction probably containing GMP was eluted by 1 litre of 1.0 M NaCl in 0.05 M phosphate buffer solution at pH 7.0. The eluate and defatted whey were then analysed by SDS-PAGE.

Fig. 5-13 Spectrophotometric Pattern of the eluate after Treatment with 12% TCA. TCA was added to the eluate from IRA 93 to make 12% concentration, and the precipitate was removed by filter paper No.541. TCA and NaCl were removed from the supernatant by dialysis against distilled water.
Table 5-5
Amino Acid Analysis of TCA-treated Eluate from Cheddar Cheese Whey

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Theoretical Value</th>
<th>Experimental Value of Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( residues / mol.)</td>
<td></td>
</tr>
<tr>
<td>Asx (D/N)</td>
<td>4.00</td>
<td>3.76</td>
</tr>
<tr>
<td>Glx (Q/E)</td>
<td>10.00</td>
<td>9.71</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>6.00</td>
<td>5.91</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>1.00</td>
<td>1.49</td>
</tr>
<tr>
<td>His (H)</td>
<td>0.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>0.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>11.00</td>
<td>10.73</td>
</tr>
<tr>
<td>Ala (A)</td>
<td>6.00</td>
<td>5.27</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>8.00</td>
<td>8.41</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>0.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>Val (V)</td>
<td>6.00</td>
<td>5.55</td>
</tr>
<tr>
<td>Met (M)</td>
<td>1.00</td>
<td>0.92</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>7.00</td>
<td>6.33</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>1.00</td>
<td>2.65</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>0.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>3.00</td>
<td>3.30</td>
</tr>
</tbody>
</table>

*Theoretical Values are based on the primary structure of GMP.

Single letter codes are bracketed. n.d.: not detected.

---

Fig. 5-14 Analysis for Amino Acid Sequence of TCA-treated Eluate from Cheddar Cheese Whey

( I ) Amino Acid Sequence of GMP of k-casein B (Sleigh, 1982):

106

MAIPPKKNQDKTEIPTINTIASGEPTSTPTIE

137

AVESTVATLEASPVEIESPPPEINTVQVTSTAV

( II ) Sequenced Fragment of TCA-treated Eluate:

1

MAIPPKKNQDKTEIPTINTIASGEPTSTPTIE

32

AVESTV---------
Procedure A - Isolation of Igs from Pretreated Cottage Cheese Whey:

- Retentate of Pretreated Whey by UF in 10 kD
- Treated by Anion Exchanger of IRA 93 at ca. pH 7.0
- A fraction going through the resin contains Igs
- Treated by Cation Exchange
- Adsorption of Igs
- A fraction going through the resin contains La, Lg and BSA
- A fraction containing Igs
- Ultrafiltration by 150 kDa cut-off membrane
- Igs-enriched fraction
- Dry
- Igs or major whey proteins powders

Fig. 5-15a Process for Isolation of Igs and GMP from Cheese Whey. The procedures with dotted-lines are optional.
Procedure B Isolation of Igs from Pretreated Cheddar Cheese Whey:

Retentate of Pretreated Whey by UF in 10 kD

Adsorption of GMP and a GMP fraction

Treated by Anion Exchanger of IRA 93 at pH 4.7

Whey going through the resin

Adsorption of La, Lg and BSA onto the resin

A fraction containing major whey proteins such as La, Lg and BSA

Treated by Anion Exchanger of IRA 93 at ca. pH 7.0

A fraction going through the resin contains Igs

Ultrafiltration by 150 kDa cut-off membrane

A fraction containing Igs

Demineralization

Dry

Igs, GMP or major whey proteins powders

Fig. 5-15b Process for Isolation of Igs and GMP from Cheese Whey. The procedures with dotted-lines are optional.
ISOLATION AND CHARACTERIZATION OF
COMPONENTS FROM WHEY

BY
YUE XU

A thesis submitted in partial fulfilment of the requirements for the degree of
DOCTOR OF PHILOSOPHY
School of Food Science
UNIVERSITY OF WESTERN SYDNEY, HAWKESBURY

Volume II

APRIL, 1996

Supervisory Panel
Assoc. Prof. Jim Hourigan, University of Western Sydney, School of Food Science
Dr. Robert Sleigh, Australian CSIRO, Division of Food Science and Technology
Dr. Bob Johnson, Australian CSIRO, Division of Food Science and Technology
The candidate, Yue Xu, hereby declares that this submission is his own work except where due acknowledgment is made in the text, and that none of the work in this thesis has been submitted to any other university or institution for a higher degree.

Yue Xu
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Chapter 6

Separation of Lactose by Ion Exclusion Chromatography

ABSTRACT

Ion exclusion chromatography, developed by Wheaton & Bauman (1953), has been used for the separation of salt (CaCl₂ or NaH₂PO₄) and lactose in this study.

Eight strong cation and anion exchange resins, each with a polystyrene matrix, and their salt forms including ammonium (NH₄⁺), sodium (Na⁺), potassium (K⁺), calcium (Ca²⁺), chloride (Cl⁻), carbonate (CO₃²⁻), sulphate (SO₄²⁻), and phosphate (PO₄³⁻) were screened by using the model solutions containing salt and lactose. The selectivity factor (α) and elution patterns were used as the measures. The cation exchange resin PCR 553 in its NH₄-form has been found to be the optimal candidate for the separation of the model solutions. All other forms of PCR 553 when they were used for the sample containing CaCl₂ have shown poor separation.

The effects of sample pH on the separation has been investigated for both NH₄⁻ and Na-forms of PCR 553, indicating that only the solution containing lactose and NaPi shows an effect at higher pH (over pH 8.0). pH traces monitored during elution implied that the anion of the salt would be eluted in front of the cation in ion exclusion chromatography. The effect of the flow rate on the separation was as expected; the faster flow rate gave the worse separation.

Effects of the concentration and feed volume (FV) on the column efficiency, band broadening, and band shape, have been investigated by using indexes such as number of theoretical plates N, selectivity factor (α), plate height (H), and tailing factor (TF), respectively, to compare with the elution patterns.

It has been found that an improvement in column efficiency due to lengthening the column, which was indicated by rising N, is reversed by increase of the feed volume (FV). Alpha (α) value, a yardstick for the migration rate of two solutes, has indicated that the improvement of the separation between two solutes can be achieved by increasing the proportion of lactose to salt in the sample. Alpha (α) value also suggested that only with a lower FV can a longer column produce a bigger difference of the migration rate than a shorter one. When FV increases, the difference of the migration rate produced by the longer column disappeared. Serious band broadenings measured by H, have been seen by increasing the FV. Variations in the shape of the band broadening, described by TF, were greatly affected by FV rather than by the concentration.

When the results indicated by N, α, H and TF are compared with the profiles of the elutions, agreements between them are obvious. A nearly complete separation of salt and lactose can only be obtained by using 2% FV and a long column.
6.1 INTRODUCTION

The by-product of whey processing with UF, UF permeate, contains about 80% of the original lactose in whey, and the other components, including minerals, vitamins and small peptides, will pass into the permeate in various proportions (Zadow, 1984). UF permeate has a biological oxygen demand (BOD) of 30,000-45,000 mg/litre (Jelen, 1979) and cannot, therefore, be directly disposed of as waste water into sewage. For many years, lactose was mainly produced directly from whey, and in general, about 50% for the lactose in whey was recovered. More recently, permeate has become a preferred raw material for lactose (Renner & El-Salam, 1991). The permeate contains only very low molecular-weight peptides, and the crystallized lactose has better quality than that from whole whey. Thus lactose could meet the specification for a protein-free pharmaceutical grade product (Renner & El-Salam, 1991). Even so, the lactose industry is still facing the difficulty of producing very pure lactose with one step crystallization. The industrial demineralization of permeate can be easily achieved by multi-stage cation-anion exchange. However, the largest impediment to the application of ion exchangers for demineralization of such a large volume material as permeate is the cost of the chemicals required for regeneration and the disposal of the waste regenerant (Schoenrock, 1991).

Generally speaking, crystallization is a highly efficient method for fractionation or purification and has some advantages such as simplicity, low cost and so on over another powerful technology of purification — chromatography. However, crystallization has a disadvantage, namely the generation of the mother liquor. The mother liquor presents two problems in food industry; one is, it represents a significant
loss in production, and the other is, it usually contains a high BOD. Turning mother liquor into a “clean” water stream is highly costed. In lactose crystallization, the mother liquor contains nearly 50% of salt and other impurities in total solids. It could be difficult or inefficient simply to recycle the mother liquor.

Ion exclusion (IE) chromatography, which has previously been used in the separation of molasses (Schneider & Mikule, 1975) as well as dairy liquids more recently (Harju, 1987; 1989; 1990), provides a good option for separating the permeate and mother liquor into two fractions, the relatively pure lactose and the mixtures of impurities without frequent regeneration and creation of additional wastewater.

Details about the isolation of lactose by ion exclusion and analysis of ion exclusion chromatography, have been extensively reviewed in section 1.2.3 of Chapter 1 and sections 2.6.3, 2.6.4, 2.6.5 of Chapter 2, respectively.

However, since it was introduced by Wheaton & Bauman (1953) ion exclusion has been used as a practical tool, particularly in the sugar industry. Except for the early work of the column analysis for the separation between NaCl and ethylene glycol (Simpson & Wheaton, 1954), nothing has been done for the separation between sugars (sucrose or lactose) and minerals. In addition, resin types and salt forms studied for the separation of lactose (Harju, 1987) were just those adopted from the sucrose work (Hongisto, 1977; Schultz et al., 1967). When ion exclusion is used for the separation of lactose and minerals, it is necessary to widen a screening of resin types and salt forms for which are suitable, and analyse the column performance by using some mathematical indexes.
This chapter focuses on 1) selections of the resins and their salt forms suitable for the separation of lactose (non-electrolyte) and salts or peptides (electrolytes), 2) effects of some parameters on the separation of salt and lactose, 3) column analysis.

6.2 MATERIALS AND METHODS

6.2.1 Measurement of Void Volume ($V_0$)

The measurement was based on the method of Wheaton & Bauman (1953) with a slight modification. A 50 ml sample of resin was placed in a column where the level of water was adjusted to the top of the resin, then transferred in a sintered funnel and sucked by a vacuum pump to collect the interstitial water around the resin which represented void volume, $V_0$.

6.2.2 Samples and Apparatus

**Formulated Samples:**

NaH$_2$PO$_4$ and CaCl$_2$ represent major ions in permeate or mother liquor. The synthetic samples of permeate or mother liquor were made from analytical reagent of NaH$_2$PO$_4$ (Ajax) or CaCl$_2$ (BDH) plus B.P. grade lactose. In some cases, a fraction with strong absorption at 260-280 nm was made by dialysis of permeate with 2 kDa membrane tube and used as the permeate peptide.

**Columns:** A 50 ml burette, as well as glass columns of different length with jackets were used.

**Pump:** A Peristaltic Pump was used.

**Collector:** 2 ml graduated test tubes arranged in a movable rack were used as the collector.
6.2.3 Resins and Salt Forms

The resins used in ion exclusion are polystyrene-based, which are cross-linked with divinyl benzene (DVB). The effects of resin characteristics, such as degree of crosslinkage and bead size, on separation of electrolytes and sugars have been extensively studied (Wheaton & Bauman, 1953; Sayama et al., 1980). Although, generally speaking, lower crosslinkage and smaller bead size will give better separation, the resins with 3-5% DVB and 30-60 mesh (0.25-0.5 mm) are used in practice because of physical durability and efficiency. The ion exchange resins used in this study are listed in Table 6-1, PCR883 and 553 from purolite, with 4-5% crosslinkage and about 0.4 mm particle size, are typically employed in treatment of molasses.

Strong cation-exchange resins in the Na, Ca, K and NH₄-forms were prepared by contact with 4 bed volumes of 0.5 M NaCl, CaCl₂, KCl and NH₄Cl after being treated to free acidic form by 4 bed volumes 0.5 M HCl, followed by washing with distilled water to neutral pH. Cl, CO₃, SO₄ and PO₄-forms of strong anion-exchange resins were obtained by contact with 4 bed volumes of 0.5 M HCl, Na₂CO₃, Na₂SO₄ and NaH₂PO₄ after being converted to free basic form by 4 bed volumes of 0.5 M NaOH, followed by washing with distilled water.

6.2.4 Experimental Procedure

When the resin had been added to the column, proper packing of the spherical resin beads was obtained by tapping the side of the column and by passing a large volume of distilled water through the bed before use. Zonal chromatography, where the sample to be separated is injected in small quantities as a pulse onto the top of the column, was used in this study. After the level of water was adjusted to the top of the
resin, a sample of the solute mixture was carefully applied to the top of the bed. The sample was allowed to drain into the bed and was followed by the water eluent. Every 2 ml fraction was collected from the time of introducing the sample, and then analysed chemically. Different flow rates could be achieved by adjusting the tap of the column or the speed of the pump. A diagram of the experiment is shown in Fig. 6-1. Since the preliminary experiments have shown that the use of gravity or pumping force was not affecting chromatographic performance, the gravity force was mainly used to drive the liquid through the column in this study.

In the chromatographic separations of materials containing higher concentration such as more than 40% of carbohydrates (e.g. sucrose and lactose), especially in large scale, the use of higher temperature (e.g. 60-80°C) is nearly “compulsory” because the lower temperature could cause crystallization of the sugar at high concentration during chromatography and consequently clog the column. In this study, most experiments were conducted by using a lactose concentration of no more than 30%. These lactose concentrations used did not give the problem of clogging at the operation temperature of 20-24°C due to the small size of the experiment. In the experiments of varying concentrations, the lactose solution with 43 °Bx was used, where a small amounts of warmed sample (about 60-70°C) were applied to the column until drained into the resin bed.

6.2.5 Parameters used or tested:

**Resin Bed Volume:** 50 ml, 150 ml

**Salt Forms of Resins:** Cl, CO₃, SO₄ and PO₄-forms were chosen for strong anion exchange resins. Strong cation exchange resins had Na, Ca, K and NH₄-forms.
Flow Rate: 1 ml/min and 4 ml/min.

Temperature: 20-25°C

Feed Volume: 2%, 14%, 30% and 50% of resin beds.

Feed Concentration: 5°Bx, 15°Bx, 30°Bx and 43°Bx of lactose, as well as 0.01 M, 0.05 M, 0.1 M and 0.25 M CaCl₂ and NaH₂PO₄.

Length of Resin Bed: 40 and 53 cm with a diameter of 10 mm and 110 cm with a diameter of 12 mm.

6.2.6 Mathematical Analysis

Distribution Coefficients ($K_d$):

As discussed in Chapter 2, $K_d$ can be expressed as in equation (6-1) (Simpson & Wheaton, 1954):

$$K_d = \frac{V_m - V_o}{V_r}$$

(6-1)

If molar concentration is introduced, $K_d$ can be changed to the Capacity Factor $k'$ defined as in equation (6-2) (Small, 1989):

$$k' = \frac{V_m - V_o}{V_o}$$

(6-2)

Wheaton & Bauman (1953) have shown that each solute has a definite $K_d$ when placed in contact with ion exchangers and that these $K_d$ values will vary with different resins as well as with the different ionic forms of these resins. The electrolyte is excluded from the interior of the resin bead and the non-electrolyte is not, thus producing a difference in the distribution of the electrolyte and the non-electrolyte between the resin and solution phases. A difference in $K_d$ means that a separation of solutes is possible and can therefore be used to determine the separability of the solutes. The $K_d$ can also be used to determine the rate of travel of a given solute, and consequently the order in which a group of solutes will travel down an ion exclusion
column. This then means that the order of elution can be predicted from the $K_d$ value. The solute having the lowest value of $K_d$ will appear in the effluent first. In the case of an aqueous solution of ionic and non-ionic solutes, the ionic material appears in the effluent prior to the non-ionic.

$k'$ is an experimentally more accessible term but a less fundamental factor than $K_d$ (Small, 1989). However, both $K_d$ and $k'$ represent the same characteristics of the retention in ion exclusion chromatography. For convenience, $K_d$ is described by the value of $k'$ in this study. In practice, when $V_m$ is determined from an elution graph of the experiment, $k'$ is easily obtained by equation (6-2).

$V_o = \text{liquid volume in external void space of a resin bed (Void Volume)}$

$V_r = \text{volume of liquid held within resin particles}$

$V_m = \text{volume of effluent from the point introducing the sample to the maximum peak on elution graph}$

**The Selectivity Factor ($\alpha$):**

The *Selectivity Factor* (Skoog & Leary, 1992), $\alpha$, is used to describe the differential migration rate between non-ionic material (lactose) and ionic materials (salts or peptides), which is defined as in equation:

$$\alpha = k'^L / k'^I$$  \hspace{1cm} (6-3)

where $k'^L$ is $k'$ of Lactose, $k'^I$ is $k'$ of ionic materials such as salts and peptides.

If $\alpha$ equals 1, lactose and ionic materials will appear in the effluent together. If $\alpha$ is more than 1, ionic materials will appear in the effluent first. The larger the value of $\alpha$, the better the resolution between ionic materials and lactose.

**Plate Height ($H$) and Number of Theoretical Plates ($N$)**
The efficiency of the chromatographic column will be greatly influenced by operating parameters such as feed concentration, loading volume, column length, flow rate, and temperature. The plate theory with kinetic parameters like plate height (H) or number of theoretic plates (N) has been widely used in zonal chromatography to describe the efficiency of the column (Simpson & Wheaton, 1954; Guillaume & Guinchard, 1994).

The efficiency of a chromatographic column is described by the equation (Skoog & Leary, 1992):

\[ N = \frac{L}{H} \]  \hspace{1cm} (6-4)

H = Plate height or height equivalent to a theoretical plate in cm

N = Number of theoretical plates

L = Length of a chromatographic column (i.e. the resin bed length), in cm

The efficiency of chromatographic columns increases as the number of plates (N) becomes greater (Skoog & Leary, 1992). H is a measure of efficiency that is independent of total length (resin bed length). Because H is truly a measure of the extent of spreading of an analyte zone as it passes through a column (Miller, 1988) (i.e. band broadening), the band broadening (or zone spreading) is reduced as the plate height (H) becomes smaller (Skoog & Leary, 1992; Miller, 1988).

As given in the review (Chapter 2), there are four methods for calculation of N (Simpson & Wheaton, 1954; Khym, 1974a).

In this study, the zone-width method (Khym, 1974a) was chosen for estimation of N values, which can be computed from the equations:

\[ N = 8 \left( \frac{V_m}{\beta} \right)^2 \]  \hspace{1cm} (6-5)
where $\beta = 2W$; $W = $ Half-width (abscissa) of an elution curve at ordinate value of $1/e$ of the peak concentration ($C_{\text{max}} / e$); $e$ is the natural log base, equal to 2.718. $W$ is measured as the volume unit like $V_m$ (see Fig. 2-16 (b) of Chapter 2).

**Tailing Factor (TF)**

Band broadening can occur in three ways, e.g. symmetrically spreading, asymmetrically fronting and tailing. The tailing factor (TF) is used to describe the pattern of band broadening (Miller, 1988).

$$\text{TF} = \frac{b}{a} \quad (6-6)$$

where $a$ and $b$ are first and second half of the peak width at 10% of the height of the peak. A symmetrical peak will have a value of 1 and tailed peaks a value of greater than 1. A fronted peak (one with a leading edge and $a > b$) will have a value less than 1 (see Fig.2-20 of Chapter 2).

**6.2.7 Analytical Methods:**

**Peptides:** Absorbance at 280 nm by Perkin Elmer $\lambda$-2 spectrophotometer.

**Lactose:** Estimation of refractive index (R.I.) by Abbe refractometer. Lactose concentrations are expressed in Brix degree ($^\circ$Bx) in this study.

**Conductivity:** Activon model 301 conductivity meter.

Strictly speaking, the Brix degree is accurate only for pure sucrose solutions but is also widely used for approximate values for other solutions of sugars (Southgate, 1991). The conversion of R.I. to lactose concentrations (0.5-18%) has been tabulated in the handbook (Weast et al., 1990). In this study, the conversion of R.I. and $^\circ$Bx to lactose concentrations has been determined under conditions of this laboratory and the range of lactose concentrations tested is from 5% to 30% (Table 6-4).

**6.2.8 Compensation of Conductivity:**
The conductivity, $\kappa$ may be described as the equation (Crow, 1979):

$$\kappa = \frac{1}{\rho} = \frac{1}{RA}$$

(6-7)

$\rho$ being the specific resistance or resistivity and $l$ and $A$ the length (m) and area ($m^2$) respectively of the portion of solution studied. $1/R$ is known as the conductance of the material. $\kappa$ has the units $\mu S/cm$ or $\Omega^{-1} m^{-1}$. In practice, what is actually measured for conductivity is the resistance $R$. So, if an electrolyte solution is mixed with a non-electrolyte like lactose, the consequence is that conductivity of that system might be reduced because of enhanced resistance.

Experiments for the suppression of conductivity in the presence of lactose were conducted as follows. The stock solutions of 29.6 and 30.4 °Bx were prepared by adding 30 g lactose to 100 ml of 0.1 M NaH$_2$PO$_4$ and CaCl$_2$, respectively, then heating up to 100°C. After cooling to room temperature, the stock solutions were diluted by 0.1 M NaH$_2$PO$_4$ and CaCl$_2$ to make samples of lower Brix. Finally, samples of 0.1 M NaH$_2$PO$_4$ solutions with 0, 5.4, 11.5, 17.1, 21.5, 25.6 and 29.6 °Bx of lactose, and 0.1 M CaCl$_2$ solutions with 0, 7.0, 11.8, 17.0, 21.5, 25.6 and 30.4 °Bx of lactose were tested by the conductivity meter. The results are shown in Fig. 6-2 and 6-3. Fortunately, the decrease of conductivity of sodium phosphate and calcium chloride solutions due to addition of lactose is linear with the increase of Brix in solutions. Thus, compensation of conductivity for a lactose-containing NaH$_2$PO$_4$ or CaCl$_2$ solution can be worked out.

The slope (a) of linear curves ($\kappa_e = -a \cdot s + \kappa_i$) in Fig.6-2 and 6-3 can be easily obtained as follows.

$$a_{CaCl_2} (0.1 M) = 358.33 \ (\mu S/cm/°Bx)$$
\( a_{\text{NaPi}}\) (0.1 M) = 113.94 (\( \mu S/cm/^{\circ}Bx \))

Coefficient of compensation (\( \tilde{a} \)), defined as the reduction of conductivity by per Brix lactose, can be calculated by dividing the slope by the conductivity of the initial salt solution (lactose free). \( \tilde{a} \) after calculation is found to be constant, \( \tilde{a} = 0.019 \) \( (^{\circ}Bx^{-1}) \), for both \( \text{CaCl}_2 \) and \( \text{NaH}_2\text{PO}_4 \) though initial solutions of \( \text{CaCl}_2 \) and \( \text{NaH}_2\text{PO}_4 \) are very different in conductivity.

Thus, calibrated conductivity (\( \kappa_c \)) of 0.1 M \( \text{NaH}_2\text{PO}_4 \) or \( \text{CaCl}_2 \) solution containing lactose can be obtained by reading conductivity (\( \kappa_r \)) plus a compensation (\( \tilde{a} \cdot s \cdot \kappa_i \)).

\[
\kappa_c = \kappa_r + \tilde{a} \cdot s \cdot \kappa_i \tag{6-8}
\]

Since the calibrated conductivity (\( \kappa_c \)) in a lactose-containing salt solution should be equal to the conductivity (\( \kappa_i \)) in that salt solution with lactose free, \( \kappa_i \) is then replaced by \( \kappa_r = \kappa_c \), equation (6-8) can be rewritten as follow:

\[
\kappa_c = \kappa_r / (1 - \tilde{a} \cdot s) \tag{6-9}
\]

\( \kappa_c \) = real conductivity after calibration (\( \mu S/cm \))

\( \kappa_r \) = conductivity of the fractions (\( \mu S/cm \))

\( \kappa_i \) = conductivity of initial salt solution with lactose free (\( \mu S/cm \))

\( \tilde{a} \) = compensation coefficient, 0.019 \( (^{\circ}Bx^{-1}) \)

\( s \) = lactose concentration \( (^{\circ}Bx) \)

\( a_{\text{CaCl}_2} \) = coefficient of 0.1 M \( \text{CaCl}_2 \) solution, 358.33 (\( \mu S/cm/^{\circ}Bx \))

\( a_{\text{NaPi}} \) = coefficient of 0.1 M \( \text{NaH}_2\text{PO}_4 \) solution, 113.94 (\( \mu S/cm/^{\circ}Bx \))

Equation (6-9) was used for calibration of the conductivity in lactose-containing salt fractions in chromatography. The values of \( a_{\text{CaCl}_2} \) and \( a_{\text{NaPi}} \) listed above can be used
only for 0.1 M CaCl₂ or NaH₂PO₄ solution. The value of $a_{\text{salt}}$ must be accurately remeasured when the concentration or nature of the salt solution is changed.

6.3 RESULTS AND DISCUSSIONS

6.3.1 The Screening of Ion Exclusion Resins and their Forms by $k'$ and $\alpha$

Since Wheaton and Bauman (1953) discovered ion exclusion chromatography, the effects of the properties of the resin and its salt form on ion exclusion have been extensively studied for separations of ethylene glycol and NaCl (Simpson & Wheaton, 1954), sugar (or glucose) and salt (Hongisto, 1977; Schultz et al., 1967), glucose and fructose (Ghim & Chang, 1982; Dechow, 1991). As it has been suggested by these studies, the preferred crosslinkage of the resin is between 2.0 and 6.0%. In actual fact the resin having crosslinkage between 3.0 and 5.0% is used for separation purposes (Hutchinson & Katsahian, 1980). The lower the crosslinkage the better the separation of the two solutes, however lower crosslinked resins are less stable than higher crosslinked resins, so, consequently, the actual resin used will be a compromise of stability over the degree of separation. As compared with macroporous resins, the gel resins have a lower glucose (sugar) loss and have less NaCl in the product (Dechow, 1991). The salt forms commonly used in ion exclusion are $K^+$, $Na^+$ and $Ca^{2+}$. The salt forms of $SO_3^{2-}$, $CO_3^{2-}$, $H_2PO_4^-$, $Sr^{2+}$ and $Zn^{2+}$ have also been tried for the separation of glucose and fructose (Ghim & Chang).

In this study, the PCR 553 and 833 resins of the Purolite company, designed for liquid sugars, have been chosen as the preferred resins for the separation of salt and lactose. Resins with higher crosslinkage, macroporous, and anion exchange resins have
also been studied in case they show better separation of salt and lactose. The properties of screened resins are listed in Table 6-1. The selectivity factor (α), which describes differential rates of migration of two solutes in chromatography, was used as the screening criterion for the resins of ion exclusion, and the results are shown in Table 6-2. As it has been stated above, the bigger the value of α above 1.0, the better the separation between ionic and non-ionic material. Among 8 resins tested, PCR 553, PCR 833, and IRA 401 give bigger α values which are 1.47, 1.47 and 1.53 for the sample of lactose and NaPi and 1.12, 1.12 and 1.21 for the sample of lactose and CaCl₂, respectively. Neither macroporous and macroreticular (150C, IRA 900, IRA 938) nor higher crosslinkage (Dowex 2) resins of both cation and anion exchange have shown better separation of salt and lactose. The effect of different forms of resins PCR 553 and IRA 401 on the separation of salt and lactose is shown in Table 6-3 and Fig. 6-4. The salt forms investigated in this study include NH₄⁺, Na⁺, K⁺ and Ca²⁺ for cation exchange resin (PCR 553) and Cl⁻, CO₃²⁻, SO₄²⁻, and PO₄³⁻ for anion exchange resin (IRA 401). K and Na forms were most commonly used in ion exclusion to treat molasses (Schoenrock, 1991; Schneider, 1975; Hongisto, 1977; Schultz et al., 1967; Dechow, 1991) and dairy streams (Harju, 1989; Harju, 1990), whereas the Ca-form of the resin is preferred in separation of glucose and fructose (Dechow, 1991). When the Ca-form of the resin was used in the purification of sucrose from molasses, poor separation between salt and sugar was obtained (Reents & Keller, 1960; Schoenrock, 1991). Such a poor performance of the Ca-form on purification of sucrose from molasses was explained (Schoenrock, 1991) as a decrease of resin pore size, due to multivalent cation exchange, which apparently restricted sucrose penetration. The Ca-form of PCR 553 used for the separation of salt and lactose here, gave a similar result,
where both $\alpha$ values (Table 6-3) and elution patterns (Fig. 6-4 (6)) showed no resolution between salt and lactose at all. Of all cation forms tested, the NH$_4$-form has shown the best separation based on both $\alpha$ values ($\alpha = 1.25$) and elution patterns (Fig. 6-4 (1), (2)).

The salt forms of the anion exchange resin (IRA 401) have also been investigated (Table 6-3 and Fig. 6-4). The SO$_4$ and PO$_4$ forms ($\alpha$ around 1.0) can be ruled out as the candidates for the isolation of lactose in the salt solution. Although the CO$_3$ and Cl forms have given fairly good $\alpha$ values of ($\alpha = 1.26$ and 1.24) in comparison with $\alpha$ 1.25 for the NH$_4$-form of PCR 553, the elution patterns of these anion forms can not match their $\alpha$ values. Because the selectivity factor ($\alpha$) is calculated from the position of maximum solute eluted, any other factors such as band broadening, stronger adsorption etc. will result in disagreement between $\alpha$ and the real profiles of chromatography. The CO$_3$ form of IRA 401 probably desorbed or underwent a strong exchange with Pi (Fig. 6-4 (12)) as indicated by much higher salt in eluates (Ce/Cf > 2) while the CO$_3$ form of IRA 401 can adsorb Ca$^{2+}$ very strongly (Fig. 6-4 (13)) which behaviour could be suitable for other applications but not for ion exclusion. On the other hand, the Cl-form caused the bands of both NaPi and CaCl$_2$ to give tail broadening and to emerge as shoulders (Fig. 6-4 (9), (10)), resulting in the increase of overlapped fractions of salt and lactose.

The peptides of whey permeate, on the one hand, have been eluted in a similar way to ionic material on PCR 553, namely they have been excluded (Fig. 6-4 (5)). On the other hand, these peptides are irreversibly adsorbed by IRA 401 when water is used as eluate (Fig. 6-4 (11)).
Except for the NH$_4$-form, all other forms of PCR 553 when they were loaded with CaCl$_2$ have shown relatively poor separation. In general, multivalent cations such as Ca$^{2+}$ will give poor resolution in ion exclusion system because of a rapid decrease of resin pore size due to replacement of the monovalent cation form of the resin by Ca$^{2+}$ (Reents & Keller, 1960). This apparently restricts penetration of non-ionic material such as sucrose. Another reason is probably that multivalent cations replace monovalent cation, and then penetrate into the resinous phase to be commingled with the sugar's entry (Schoenrock, 1991).

The equilibrium of cation exchange reaction of monovalent (M$^+$) and Ca$^{2+}$ can be expressed as follows (Khym, 1974b):

$$2\text{RSO}_3^- \text{M}^+ + \text{Ca}^{2+} \rightleftharpoons (\text{RSO}_3^-)_2\text{Ca}^{2+} + 2\text{M}^+ \quad (6-10)$$

$$K_Q = \frac{[(\text{RSO}_3^-)_2\text{Ca}^{2+}][\text{M}^+]^2}{[\text{RSO}_3^- \text{M}^+]^2 [\text{Ca}^{2+}]} \quad (6-11)$$

$K_Q$ (Khym, 1974b) is a measure of the exchange power of a resin for one particular ion relative to another. In fact, the value of $K_Q$ is often referred to as the selectivity coefficient of a resin, and if this index of resin affinity is determined for a series of ions, one then has the data to establish a selectivity scale. Based on $K_Q$ for the exchange of several cations on sulfonated polystyrenes (Dowex 50) with respect to hydronium ion as reference (Small, 1989a), the affinity of the alkali metal ions for this type of resin displays a preference in the order:

$$\text{H}^+ < \text{Na}^+ < \text{NH}_4^+ < \text{K}^+ < \text{Mg}^{2+} < \text{Ca}^{2+}$$

As Ca$^{2+}$ has a stronger affinity for the resin of sulfonated polystyrenes than the cations that were used as the salt form in this study, displacement of those monovalent cations
by \( \text{Ca}^{2+} \) inevitably occurs when ion exclusion resin is loaded with the divalent ion. In the treatment of molasses (Hongisto et al., 1978), if the molasses contains higher \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) concentration, the ion exclusion resin goes partly into the divalent ion forms and some of the nonsugars are not separated from sucrose. This decreases the purity of the sucrose fraction. Schneider & Mikule (1975) have also demonstrated that the extraction of sugar from molasses can be carried out economically only if the exclusion resin is loaded with \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) at most to the extent of 15-20\% of its total capacity in equivalents.

However, poor separation (more overlapped) between salt and lactose has not been observed when the \( \text{NH}_4 \)-form of PCR 553 was loaded with \( \text{CaCl}_2 \) in this study (Fig. 6-4 (2)), despite replacement of \( \text{NH}_4 \) by \( \text{Ca}^{2+} \) due to cation exchange. The reason is not clear, but the difference in chemistry between \( \text{NH}_4 \) and alkali metals such as \( \text{Na}^+ \), \( \text{K}^+ \) is well understood. Firstly, \( \text{NH}_4 \) is a monovalent cation of “covalent-bonded complex” which might interact with lactose in a specific way. Secondly, \( \text{NH}_4^+ \) as a conjugate acid of weak base \( \text{NH}_3 \) can undergo dissociation reaction to a very small degree as follows:

\[
\text{NH}_4^+ \quad \leftrightarrow \quad \text{H}^+ + \text{NH}_3
\]

The small amount of \( \text{NH}_3 \), as a non-ionic compound, could penetrate into the resinous phase in the same way as lactose. Such different properties of \( \text{NH}_4^+ \) seem to help reduce band spreading of non-ionic fractions and make lactose more strongly retarded on the exclusion resin.

6.3.2 Effect of Sample pH and Flow Rate on the Separation

Fig. 6-5 and 6-6 show the effects of the different pH of samples on ion exclusion chromatography. When ion exclusion resins with either the \( \text{NH}_4^- \) or \( \text{Na}^- \)-form
were loaded with the samples of NaPi and lactose, a higher pH value of the sample gave a poorer separation (Fig. 6-5 A, B, Fig. 6-6 a, b). On the other hand, the sample pH has not exerted any influence on the separation in the case of CaCl₂ + lactose (Fig. 6-5 C, D, Fig. 6-6 c, d).

Fig.6-5 and 6-6 also give the pH traces during elution that show a similar direction of variation. In general, the pH first goes down to acidic (pH 2.5-3.5) when the salt is eluted from emergence to peak, then going up to neutral (pH 6-7) when the salt is eluted from peak to disappearance. It might be suggested that the anion of the salt is probably eluted in front of the cation in ion exclusion chromatography. However, when the PCR 553 in Na-form is loaded with the sample of NaPi at pH 8.0 (Fig. 6-6b) the pH trace is different from a general approach described above. The pH first goes down to 6-7, then immediately goes up to basic (pH 8.0) when the salt is just eluted from emergence to peak. The pH further goes up to very basic (above pH 10) when the salt peak has gone.

In general, a lower flow rate will contribute to better separation for ion exclusion chromatography of typical ionic and non-ionic solutions (Wheaton & Bauman, 1953) and molasses (Takahashi & Takikawa, 1965; Sayama et al., 1980). It is also easily understood from the theory that ion exclusion is based on partition by which solutes are separated by differences in the "solubilities" of the sample components between the stationary and mobile phases. Lower flow rates make sure that partition is optimised.

The effect of the flow rate on the separation of NaPi and lactose has been shown in Fig. 6-7, where no resolution can be made at the flow rate of 4 ml/min as compared with that at the flow rate of 1 ml/min no matter what kind of salt form of the
resin is used. However, the contribution of the flow rate to the separation is influenced by many other factors such as production scale, loading material, resin particle size, loading volume, concentration of solutes and so on. The flow rate used in practice is a compromise and will be determined specially for each case. The flow rate used in isolating lactose from dairy streams was 2.5 ml/min (150 ml/hr) (Harju, 1989).

6.3.3 Analysis for Band Broadening and Column Efficiency of Ion Exclusion

As it has been stated above, $k'$ is defined as

$$k' = \frac{\text{equilibrium quantity (moles) of solute in stationary phase}}{\text{equilibrium quantity (moles) of solute in mobile phase}}$$ (6-12)

For well-behaved chromatography (assuming no forward mixing and complete equilibrium) it is essential (Simpson & Wheaton, 1954; Knox, 1977) that $k'$ is independent of the absolute concentration in the two phases, for otherwise different parts of a solute band having different concentrations would migrate at different speeds. The consequences of this are peak tailing or fronting. In what follows it is assumed that $k'$ is indeed independent of concentration and that peaks are Gaussian in shape (Knox, 1977).

In reality, the Gaussian peak is not often obtained and it is possible that $k'$ is affected by concentration resulting in the solute migrating at different speeds. Fig. 6-8 shows the elution patterns obtained by loading with different concentrations of the solute in the sample, where small but clear differences of the solute migration are observed by varying the concentration of the solute in the loading sample. Accordingly, the values of $k'$ calculated from Fig. 6-8 show a smooth incrementation followed by raising the concentration of the solutes (Fig. 6-9). Since a bigger value of $k'$ is indicative of stronger retention (or retardation) of the solute on the resin, the
migration of the solute seems to be under the influence of the concentration. In addition to "well-behaved chromatography" not easily being achieved, slight broadening of the peak by varying the concentration is a major factor in the variation of k', an index calculated from $V_m$. Even so, one point is clear that at least the influence of the concentration on k' is not great. Thus the independence of k' on the solute concentration may be correct in principle.

The selectivity factor (α) is an index to describe differential migration rates between two species (or solutes). The separation of salt and lactose with different concentrations, expressed as α calculated from Fig.6-11, is shown in Fig.6-10. The improvement of the separation between two solutes, as indicated by the increase of the values of α, can be achieved by increasing the concentration of lactose simultaneously with reduction of the concentration of salt, namely by increasing the proportion of lactose to salt in the sample. The maximum α value of about 2.0 has been obtained from the sample containing 0.01 M CaCl$_2$ and 45°Brix lactose. On the other hand, Fig. 6-11 gives the elution patterns for the separation with varying concentration, where the reduction of the overlapped parts of the profile coincided with the increase of the α value of corresponding sample.

As it has been discussed above, k' or α is used to describe solute retention which is directly related to the thermodynamics of distribution of solutes between two zones or phases having different compositions (Knox, 1977). However, k' and α cannot give any indication of band (peak) broadening. By contrast to retention, band dispersion (broadening) in well-behaved chromatography arises exclusively from dynamic processes occurring in the column (Knox, 1977). The control of band
dispersion can therefore be considered independently from the control of retention described by \( k' \) and \( \alpha \).

Two related terms, number of theoretical plates \( N \) and plate height \( H \), are widely used as quantitative measures of chromatographic column efficiency. \( H \) is defined as the ratio of length (or height) of column \( L \) (resin bed length) to number of theoretical plates \( N \) expressed in equation (6-4).

Effect of the flow rate, or the more fundamental parameter, the linear mobile phase velocity \( (u) \) in cm/s, on \( H \) in chromatography has been extensively studied (Knox, 1977; Jones et al., 1992; Guillaume & Guinchard, 1994). The relationship of \( H \) and \( u \) is described by the van Deemter theory as follows (Skoog & Leary, 1992):

\[
H = \frac{B}{u} + Cu + A
\]

or the modernized equation of van Deemter theory (Skoog & Leary, 1992):

\[
H = \frac{B}{u} + Cu = \frac{B}{u} + (C_S + C_M)u
\]

where the quantities \( A, B, \) and \( C \) are coefficients, which are related to eddy diffusion (the \( A \) term), longitudinal molecular diffusion (the \( B \) term) and mass transfer in the stationary phase (the \( C \) term), respectively. The \( C \) term is split into two parts in the modernized version, \( C_S \) for mass transfer in the stationary phase and \( C_M \) for mass transfer in the mobile phase. A plot of \( H \) versus \( u \) gives a curve in which at low \( u \) the \( B \) term dominates and is responsible for the rising value of \( H \) as the velocity falls; at high \( u \) the \( C \) term dominates and is responsible for the rise of \( H \) as the velocity rises (Knox, 1977).

The effect of the flow rate (gallons per square foot per minute) on \( H \) was measured for ion exclusion chromatography (Simpson & Wheaton, 1954), where probably because the flow rate used was high enough so that only the \( C \) term
dominated, the rise of H (more dispersion of the band) was observed as the flow rate rose. The particle diameter of the resin is another factor that greatly affected H; the value of H increased linearly with enlargement of the particle diameter of the resin (Simpson & Wheaton, 1954). In addition, the relationship of temperature and H were also examined in chromatography (Jones et al., 1992; Guillaume & Guinichard, 1994). In gas chromatography, an increase in temperature produces a decrease in H (Jones et al., 1992) whereas in liquid chromatography (e.g. HPLC) the decrease of H with rising temperature occurred at a relatively higher flow rate (Guillaume & Guinichard, 1994), such as 1 ml/min.

In this study, the effects of the concentration and feed volume on number of theoretical plates N and plate height H have been studied. It is well known that there are many methods that can be used for the determination or calculation of N. So the absolute value of N could be different from various methods, but the relative tendency indicated by different N values is the same. The zone-width method (Khym, 1974a) expressed in equation (6-5), was chosen for estimation of N values in this study because of its similarity to the equation (equation 2-41 in Chapter 2) widely used in HPLC (Knox, 1977; Guillaume & Guinichard, 1994).

Fig. 6-12 shows the profiles of elution by varying feed volume (FV) in different resin bed lengths (RBL). The feed volume is critical for the band broadening which is displayed in much greater degree by varying the FV than by varying the concentration (Fig. 6-8). In the plate theory, the resin bed in a column is hypothetically subdivided into a number of plates that are operated at equilibrium (Small, 1989b). So, it is understandable that N will be increasing with the lengthening of RBL (higher column efficiency). However, this study has indicated that the increase of N due to
RBL is dependent of FV as shown in Fig. 6-13. When FV is used in 2% of RBV, N values of 241 and 170 for salt and lactose, respectively, are obtained from 110 cm RBL (Ratio_{D/D} is 92) compared with the corresponding N values of 127 and 65 from 53 cm RBL (Ratio_{D/D} is 53). When FV used increases to 14% and more, N values of 110 cm RBL dropped dramatically while the N values of 53 cm RBL are slightly decreased, and eventually N values of 110 and 53 cm RBL are very close at high FV. The result has suggested that by using FV more than 14% of RBV little improvement of column efficiency can be achieved by lengthening RBL. Even if maximum 241 number of theoretical plates can be obtained with the ratio_{D/D} of 92 in 2% FV, this N value is still very much lower than that of 7,000 - 10,000 from HPLC (Knox, 1977). Certainly, it is impossible that a chromatographic technique designed for industrial application like ion exclusion here, can match the efficiency of HPLC — a powerful analytical tool, but the comparison indicates a great disparity of the column efficiency.

A very similar trend of separation efficiency by varying FV is indicated by examination of the selectivity factor, $\alpha$ (Fig.6-14). The $\alpha$ values of 53 and 110 cm RBL with loading of 2% FV are 1.6 and 2.6, respectively and they did not change on loading with 14% FV and above as much as with 2% FV. As the higher the value of $\alpha$ the better the separation is, small feed volumes combined with increasing RBL contributes significantly to the excellent separation of salt and lactose (Fig. 6-18, (I)).

The effects of varying FV on plate height (H) — an index to measure the band broadening — are given in Fig.6-15. Loaded with 2% FV, the columns with 53 and 110 cm RBL produce smaller H with close values around 0.42 - 0.82 cm. As more than 14% FV are loaded, all values of H increase while the sharpest rise is observed in the case of 110 cm RBL loaded with 50% salt. In the case of loading 50% FV, the
values of H in 110 cm RBL are 13.34 cm (salt) and 9.80 cm (lactose) as compared with that in 53 cm RBL as 6.71 and 4.64 cm for salt and lactose, respectively. The trends of the curves in Fig. 6-15 have suggested that increasing the FV of ionic matter (NaPi) and lengthening the resin bed, lead to large increments of H (i.e. more band broadening). General speaking, ionic matter is eluted ahead of non-ionic matter in ion exclusion chromatography, and in most cases the profiles of elution show partially overlapped peaks. To achieve a reduction of overlapping, more band broadening is preferred in the lactose peak rather than the salt peak and gives rise to obtain more pure parts of lactose fraction. However, as opposed to this, increasing feed volume has actually widened the band broadening of the salt peak. Fig. 6-16 has compared the effect of concentration with that of feed volume on plate height under conditions of fixed 53 cm of RBL. In the case of increasing the concentration (Fig. 6-16 A, B), little increase of H has been seen in salt elutions, whereas a steady increase of H occurs in lactose elution. On the other hand, the sharp increases of H for both salt and lactose elutions have been obtained by increasing the feed volume (Fig. 6-16 C, D).

So far, the relationships of the concentration and FV to the band broadening measured by H have been discussed. But, we have no idea of the shape of the band broadening. Band broadening occurs in three ways (shapes), e.g. symmetrically spreading, asymmetrically fronting, and tailing. An index, the tailing factor (TF), is introduced here to describe the shape of band broadening (Miller, 1988). A symmetrical shape will have a value of 1 and tailed shapes a value of greater than 1. A fronted shape will have a value less than 1. Variations of TF values by varying the concentration and the feed volume have been shown in Fig. 6-17. On varying the concentration (Fig.6-17A, curves I and II), the TF values of salt remain steady in less
than 1 (fronting shape) whereas the elutions of lactose give the TF values more than 1 (tailing shape), indicating that the concentration exerts little effect on the shape of the elution band. In the case of FV (Fig. 6-17B, curves III and IV), the values of TF have undergone dramatic change in the wake of increasing FV, and the direction is changed from more than 1 in 2% FV to less than 1 in 50% FV, suggesting that FV has a great effect on the shape of the elution band which is shifted from tailing in lower FV to fronting in higher FV. This might also imply that the increase of the feed volume tends to backward mixing of the solutes in chromatography.

In the lowest and highest FVs, the elutions of lactose and salt have the same shapes of band broadening, i.e. the tailing in 2% FV or fronting in 50% FV. But, in 14% and 30% FV, the elutions of salt and lactose form different shapes of band broadening with the fronting and tailing, respectively, showing the shape of broadening consistent with that of varying concentration (Fig. 6-17A, curves I and II) in which 30% FV is used. In addition, by comparison of the TF values with the profiles in Fig.6-8, 6-12 (A) and (B), on the whole, the shapes of broadening suggested by the calculated index like TF agree with that of the real profiles.

The elution patterns for the separation of salt and lactose by varying the feed volume are given in Fig. 6-18. If the area of overlapping in the profiles is regarded as a measure of the efficiency of separation, the area of the overlapped part is enlarged, namely the efficiency of separation deteriorated, with the increase of an feed volume, in agreement with the result given by such the quantitative indexes as a, N, H and TF. After summing up all the profiles in this study, nearly complete separation can only be obtained by using 2% FV and longer columns of 110 cm RBL (Fig. 6-18, I). However, loading of 2% FV gives a lactose fraction that is very much diluted.
6-4 CONCLUSION

This chapter has focused on aspects including selection of resins and their salt forms, effect of some parameters on separation, and column analysis.

Eight strong cation and anion exchange resins of polystyrene were screened by using the model solutions containing salt and lactose. The selectivity factor ($\alpha$) and elution patterns were used as the measures. Cation exchange resin PCR 553 used in sugar processing, has been found to be the best resin for the separation of lactose and salt.

Different salt forms of PCR 553 and IRA 401 including $\text{NH}_4^+$, $\text{Na}^+$, $\text{K}^+$, $\text{Ca}^{2+}$, $\text{Cl}^-$, $\text{CO}_3^{-}$, $\text{SO}_4^{2-}$, and $\text{PO}_4^{3-}$ have been compared by using $\alpha$ and the elution patterns, and the best salt forms for cation and anion exchange resins are $\text{NH}_4^+$ and $\text{Cl}^-$, respectively. Poor performance of the Ca-form is observed for the separation of salt and lactose in this study, in agreement with that for the molasses process in previous reports (Dechow, 1991; Reents & Keller, 1960). Na and K-forms are the popular forms for the sugar process (Schoenrock, 1991; Dechow, 1991) whereas mainly Na-form is used in the lactose fractionation (Harju, 1989; 1990). Used in the case of lactose, the K-form has shown worse separation than the Na-form which, on the other hand, gives a poorer performance than $\text{NH}_4^+$. In addition, except $\text{NH}_4$-form, all other forms of PCR 553 when they were loaded with $\text{CaCl}_2$ have shown relatively poor separation. The Cl-form of IRA 401 is suggested by $\alpha$ value as a good form for the separation of lactose, which however, doesn’t agree with the results from the elution patterns due to the tailing dispersion of the salt peak. In addition, irreversible
adsorption of peptides and coloured compounds by IRA 401 matrix are observed when water is used as eluate, which is not acceptable in an ion exclusion system.

The cation exchange resin, PCR 553, was eventually selected for its advantage over others. The effects of sample pH and flow rate on the separation have been investigated for both the NH₄⁺ and Na-forms of the resin, indicating that only the solution containing lactose and NaPi shows an effect at higher pH (over pH 8.0). In addition, pH traces monitored during elution have implied that the anion of the salt would be eluted in front of the cation in ion exclusion chromatography. The effect of the flow rate on the separation is as expected: a fast flow rate worsened the performance of ion exclusion.

The column efficiency, band broadening and band shape have been investigated by using the indexes of N and α, H, and TF, respectively. Effects of the concentration and feed volume (FV) have been examined in this study.

The improvement of the separation between two solutes, as indicated by the increase of the values of α, can be achieved by increasing the concentration of lactose simultaneously with reduction of the concentration of salt, namely by increasing the proportion of lactose to salt in the sample.

It is common knowledge that the number of theoretical plates N will increase (improvement of the column efficiency) with the lengthening of the column (resin bed). However, this study has found that the increments of N due to lengthening the column is offset by the increase of the feed volume. α values, a yardstick for the migration rate of two solutes, also suggest that only at lower FV can longer columns produce a bigger difference in the migration rate than shorter ones. When FV increases, the difference of the migration rate produced by the length of the column disappeared. Serious band
broadenings measured by *plate height* H have been seen by rising FV. The shape of the band broadening, described by *the tailing factor* TF, is greatly affected by FV. In the lowest and highest FVs, the elution patterns of lactose and salt have the same shapes of band broadening, i.e. tailing in 2% FV or fronting in 50% FV. But, in 14% and 30% FV, the elution patterns of salt and lactose form different shapes of band broadening with the fronting and tailing, respectively.

On the other hand, varying concentration has exerted little effect on the band broadening and the shape. The results of varying concentration on \( \alpha \) have suggested that a higher concentration of loading is beneficial to ion exclusion and the sample containing more lactose and less salt will give best separation.

When the results indicated by N, \( \alpha \), H and TF are compared with the profiles of the elutions, agreements between them are obvious. A nearly complete separation of salt and lactose can only be obtained by using 2% FV and long columns.

From this study, such general operating conditions of ion exclusion for a better separation between lactose and minerals might be concluded as higher concentration of loading sample, smaller loading volume, longer column, lower flow rate and, of course, higher operating temperature since higher concentration has been used. So, for a large scale operation, continuous chromatography is better to be used for ion exclusion. For the future research, the continuous simulated moving bed (SMB) technology that has been used in de-sugarization of cane molasses (Saska & Lancrenon, 1994), should be studied for the separation of lactose from permeate and mother liquor.
6-5 NOMENCLATURE

\( \alpha \) = the selectivity factor for differential migration rate of two solutes.

\( C_e \) = concentration of the solute in effluent.

\( C_f \) = concentration of the solute in feed solute.

\( F_V \) = the feed volume.

\( F_R \) = the flow rate.

\( H \) = the plate height, cm.

\( HPLC \) = high performance liquid chromatography.

\( K_d \) = distribution coefficient

\( k' \) = capacity factor

\( K_Q \) = the selectivity coefficient of an ion on ion exchanger

\( N \) = number of theoretical plates.

\( RBL \) = the resin bed length, cm.

\( RBV \) = the resin bed volume, ml.

\( Ratio_{L/D} \) = the ratio of length to diameter of the resin bed.

\( SF \) = salt form.

\( TF \) = the tailing factor.

\( V_o \) = liquid volume in external void space of a resin bed (Void Volume).

\( V_r \) = volume of liquid held within resin particles

\( V_m \) = volumes of effluent from point introducing sample to maximum peak on elution graph.

\( Brix \) = \(^{\circ}\)Bx, the per cent of sugar (lactose) indicated on Abbe refractometer
6.6 REFERENCES


APPENDIX 6-1: TABLES
IN CHAPTER 6
### Table 6-1 Properties of Screened Ion Exchange Resins

<table>
<thead>
<tr>
<th>Resin Name</th>
<th>Ion Exchange Type</th>
<th>Structure</th>
<th>Matrix</th>
<th>Functional Group</th>
<th>Crosslinking</th>
<th>Bead Size</th>
<th>Exchange Capacity, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>*PCR 553</td>
<td>Strong Cation</td>
<td>Gel Type</td>
<td>Styrene DVB</td>
<td>Sulphonic Acid</td>
<td>4%</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>*PCR 833</td>
<td>Strong Cation</td>
<td>Gel Type</td>
<td>Styrene DVB</td>
<td>Sulphonic Acid</td>
<td>5%</td>
<td>333</td>
<td></td>
</tr>
<tr>
<td>*150C</td>
<td>Strong Cation</td>
<td>Macroporous</td>
<td>Styrene DVB</td>
<td>Sulphonic Acid</td>
<td>450-500</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>#IRA 401</td>
<td>Strong Anion</td>
<td>Gel Type</td>
<td>Styrene DVB</td>
<td>Quat Amine</td>
<td>4%</td>
<td>400-500</td>
<td>0.8</td>
</tr>
<tr>
<td>#IRA 402</td>
<td>Strong Anion</td>
<td>Gel Type</td>
<td>Styrene DVB</td>
<td>Quat Amine</td>
<td>4%</td>
<td>430-510</td>
<td>1.25</td>
</tr>
<tr>
<td>#IRA 900</td>
<td>Strong Anion</td>
<td>Macro-recticular</td>
<td>Styrene DVB</td>
<td>Quat Amine</td>
<td>430-510</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>#IRA 938</td>
<td>Strong Anion</td>
<td>Macro-recticular</td>
<td>Styrene DVB</td>
<td>Quat Amine</td>
<td>300-400</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>@Dowex 2</td>
<td>Strong Anion</td>
<td>Gel Type</td>
<td>Styrene DVB</td>
<td>Quat Amine</td>
<td>8%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The Purolite Company and Purolite International Limited; # Rohm and Hass Company;
@ Dow Chemical Company.

### Table 6-2 Screening of Ion Exclusion Resins for Separation of Salt and Lactose by k' and α

<table>
<thead>
<tr>
<th>Resin Name</th>
<th>PCR 553</th>
<th>PCR 833</th>
<th>*150C</th>
<th>#IRA 401</th>
<th>#IRA 900</th>
<th>Dowex 2</th>
<th>#IRA 402</th>
<th>#IRA 938</th>
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<tbody>
<tr>
<td>a V₀</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>16</td>
<td>20</td>
<td>20.25</td>
</tr>
<tr>
<td>a Vₘ (Lac)</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>40</td>
<td>38</td>
<td>30</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>a Vₘ (NaPi)</td>
<td>30</td>
<td>30</td>
<td>34</td>
<td>32</td>
<td>40</td>
<td>30</td>
<td>32</td>
<td>48</td>
</tr>
<tr>
<td>a Vₘ (Ca)</td>
<td>34</td>
<td>34</td>
<td>36</td>
<td>36</td>
<td>42</td>
<td>30</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>k' (Lac)</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.35</td>
<td>1.24</td>
<td>0.88</td>
<td>0.7</td>
<td>1.37</td>
</tr>
<tr>
<td>k' (NaPi)</td>
<td>0.76</td>
<td>0.76</td>
<td>1</td>
<td>0.88</td>
<td>1.35</td>
<td>0.88</td>
<td>0.6</td>
<td>1.37</td>
</tr>
<tr>
<td>k' (Ca)</td>
<td>1</td>
<td>1</td>
<td>1.12</td>
<td>1.12</td>
<td>1.47</td>
<td>0.88</td>
<td>0.7</td>
<td>1.37</td>
</tr>
<tr>
<td>α (Lac/NaPi)</td>
<td>1.47</td>
<td>1.47</td>
<td>1.12</td>
<td>1.53</td>
<td>0.92</td>
<td>1</td>
<td>1.17</td>
<td>1</td>
</tr>
<tr>
<td>α (Lac/Ca)</td>
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<td>1.12</td>
<td>1</td>
<td>1.21</td>
<td>1.04</td>
<td>1</td>
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Chromatographic condition: RBL = 40 cm, RBV = 50 ml, FV = 30% of RBV, FR = 1 ml/ml, Temperature = 20 - 24 °C, Sample: 30 Bx lactose + 0.1 M NaH₂PO₄ or CaCl₂. All resins were in Na-form.
Lac: Lactose, NaPi: NaH₂PO₄, Ca: CaCl₂.

a ml unit.
### Table 6-3 Selection of Salt Forms for Resin PCR 553 and IRA 401 by k' and α

<table>
<thead>
<tr>
<th>Resin</th>
<th>Salt Form</th>
<th>k' (Lac*)</th>
<th>k' (NaPi*)</th>
<th>k' (Ca*)</th>
<th>α (Lac/NaPi)</th>
<th>α (Lac/Ca)</th>
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<tr>
<td>PCR 553</td>
<td>NH₄</td>
<td>1.35</td>
<td>0.88</td>
<td>0.88</td>
<td>1.25</td>
<td>1.25</td>
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<tr>
<td>PCR 553</td>
<td>Na</td>
<td>1.12</td>
<td>0.76</td>
<td>1</td>
<td>1.2</td>
<td>1.06</td>
</tr>
<tr>
<td>PCR 553</td>
<td>K</td>
<td>1.12</td>
<td>1</td>
<td>1.12</td>
<td>1.06</td>
<td>1</td>
</tr>
<tr>
<td>PCR 553</td>
<td>Ca</td>
<td>0.88</td>
<td>0.88</td>
<td>0.88</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IRA 401</td>
<td>Cl</td>
<td>1.47</td>
<td>1</td>
<td>1.12</td>
<td>1.24</td>
<td>1.17</td>
</tr>
<tr>
<td>IRA 401</td>
<td>CO₃</td>
<td>1.82</td>
<td>1.24</td>
<td>N/A</td>
<td>1.26</td>
<td>N/A</td>
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<tr>
<td>IRA 401</td>
<td>SO₄</td>
<td>1.35</td>
<td>1.35</td>
<td>1.35</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IRA 401</td>
<td>PO₄</td>
<td>1.24</td>
<td>1.12</td>
<td>N/A</td>
<td>1.06</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Chromatographic condition: RBL = 40 cm, RBV = 50 ml, FV = 30% of RBV, FR = 1 ml/ml, Temperature = 20 - 24°C, Sample: 30°Bx lactose + 0.1 M NaH₂PO₄ or CaCl₂.

* Lac: Lactose, NaPi: NaH₂PO₄, Ca: CaCl₂.

---

### Table 6-4 Refractive Index (R.I.) and Brix Degree (°Bx) of Lactose Aqueous Solution at 20°C

<table>
<thead>
<tr>
<th>A% (w/v)*</th>
<th>H% (w/v)*</th>
<th>R.I.</th>
<th>°Bx</th>
<th>A% (w/v)*</th>
<th>H% (w/v)*</th>
<th>R.I.</th>
<th>°Bx</th>
</tr>
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<tbody>
<tr>
<td>5.14</td>
<td>5.41</td>
<td>1.3401</td>
<td>4.9</td>
<td>12.86</td>
<td>13.54</td>
<td>1.3510</td>
<td>12.0</td>
</tr>
<tr>
<td>5.62</td>
<td>5.92</td>
<td>1.3410</td>
<td>5.5</td>
<td>15.00</td>
<td>15.79</td>
<td>1.3541</td>
<td>13.6</td>
</tr>
<tr>
<td>6.20</td>
<td>6.53</td>
<td>1.3421</td>
<td>6.2</td>
<td>18.00</td>
<td>18.95</td>
<td>1.3592</td>
<td>17.2</td>
</tr>
<tr>
<td>6.93</td>
<td>7.29</td>
<td>1.3430</td>
<td>6.7</td>
<td>20.35</td>
<td>21.42</td>
<td>1.3631</td>
<td>19.6</td>
</tr>
<tr>
<td>7.83</td>
<td>8.24</td>
<td>1.3442</td>
<td>7.7</td>
<td>21.93</td>
<td>23.08</td>
<td>1.3658</td>
<td>21.0</td>
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<tr>
<td>9.01</td>
<td>9.48</td>
<td>1.3461</td>
<td>9.0</td>
<td>23.75</td>
<td>25.00</td>
<td>1.3680</td>
<td>22.5</td>
</tr>
<tr>
<td>10.00</td>
<td>10.53</td>
<td>1.3478</td>
<td>9.9</td>
<td>25.91</td>
<td>27.27</td>
<td>1.3713</td>
<td>24.4</td>
</tr>
<tr>
<td>11.25</td>
<td>11.84</td>
<td>1.3491</td>
<td>10.9</td>
<td>28.50</td>
<td>30.00</td>
<td>1.3758</td>
<td>26.9</td>
</tr>
</tbody>
</table>

* A: Anhydrous Lactose; H: Lactose Monohydrate; % (w/v): g/100 ml.

The concentrations (% w/v) of lactose solutions in Table were prepared by accurately weighing amounts of lactose monohydrate then made to 10 ml in the volumetric flask by distilled water. Analytical reagent of lactose monohydrate, BDH, was used. The concentrations of anhydrous lactose were obtained by subtracting 5% of water content from lactose monohydrate.

MW of Anhydrous Lactose = 342.30; MW of Lactose Monohydrate = 360.31.
APPENDIX 6-2: FIGURES
IN CHAPTER 6
Fig. 6-1 Diagram of Chromatographic Equipments and Procedures
Fig. 6-2 Suppression of Conductivity of NaH₂PO₄ Solution by Addition of Lactose
Slope (NaH₂PO₄) = -113.94, Intercept (NaH₂PO₄) = 5959.08.

Fig. 6-3 Suppression of Conductivity of CaCl₂ Solution by Addition of Lactose
Slope (CaCl₂) = -358.61, Intercept (CaCl₂) = 18434.33.
Fig. 6-4 Elution patterns of resin PCR 553 and IRA 401 with different salt forms (I)
**Fig. 6-4** Elution patterns of resin PCR 553 and IRA 401 with different salt forms (2)
Fig. 6-4 Elution patterns of resin PCR 553 and IRA 401 with different salt forms (3)
Chromatographic conditions of Fig. 6-4: RBL=40 cm, RBV=50 ml, FV=30% of RBV, FR=1 ml/min., Temperature=20-24°C, Lactose=30 °Brix.
Fig. 6-5 Effect of the sample pH on separation by PCR 553 with \( \text{NH}_4 \)-form. Chromatographic conditions: RBL=40 cm, RBV=50 ml, FV=30% of RBV, FR=1 ml/min., Temperature=20-24°C, Lactose=30 °Brix. NaPi (NaH\(_2\)PO\(_4\)) and CaCl\(_2\) = 0.1 M.
Fig. 6-6 Effect of the sample pH on separation by PCR 553 with Na-form. Chromatographic conditions of Fig. 6-4: RBL=40 cm, RBV=50 ml, PV=30% of RBV, FR=1 ml/min., Temperature=20-24°C, Lactose=30 °Brix. NaPi (NaH₂PO₄) and CaCl₂ = 0.1 M.
Fig. 6-7 Effect of flow rate on separation by PCR-553. Chromatographic conditions: RBL = 40 cm, RBV = 50 ml, FV = 30% of RBV, Temperature = 20-24°C, Sample: 30° Brix Lactose + 0.1 M NaH₂PO₄ at pH 5.8. — Lactose, —— NaH₂PO₄.
Fig. 6-8 Elution Patterns of Varying Concentration in Loading Sample. Chromatographic conditions: Resin: PCR 553, Form: NH₄⁺, RBL=53 cm, RBV=50 ml, FV=30% of RBV, FR=1 ml/min, Temperature=20-24°C, and the warmed sample (60-70°C) of 43 Bx was used for loading. Sample pH: natural.

Fig. 6-9 Effect of Loading Concentration of Lactose and CaCl₂ on the Capacity Factor (k'). k' is calculated from Fig. 6-8.
Fig. 6-10 Effect of Concentration of Ionic and Non-ionic Material in Loading Sample on Selectivity Factor ($\alpha$). $\alpha$ is calculated from Fig. 6-11
Fig. 6-11 Elution patterns for separation of salt and lactose with varying concentration (a). Chromatographic conditions: same as Fig. 6-8. ——— CaCl₂, ——— Lactose
Fig. 6-11 Elution patterns for separation of salt and lactose with varying concentration (b).
Chromatographic conditions: same as Fig. 6-8. ——— CaCl₂, ——— Lactose
Fig. 6-12 Elution Patterns of Varying Feed Volume (FV) and Resin Bed Length (RBL). Chromatographic conditions: Resin: PCR 553, Form: NH₄, RBV = 50 and 150 ml, FR = 1 ml/min., Temperature = 20-24°C, Sample pH: Natural
Fig. 6-13 Effect of Feed Volume and Resin Bed Length on Number of Theoretical Plates (N). N is calculated from Fig. 6-12.

Fig. 6-14 Effect of Resin Bed Length and Feed Volume on Selectivity Factor (α). α is calculated from Fig. 6-18.
Fig. 6-15 Effect of Feed Volume and Resin Bed Length on Plate Height (H). Cur-1: Lactose, RBL = 110 cm; Cur-2: NaPi, RBL = 110 cm; Cur-3: Lactose, RBL = 53 cm; Cur-4: NaPi, RBL = 53 cm. H is calculated from Fig. 6-12.

Fig. 6-16 Effect of Concentration and Feed Volume on Plate Height (H). Curve A: varying concentration of CaCl₂, Curve B: varying concentration of lactose, Curve C: varying feed volume of 0.1 M NaPi, Curve D: varying feed volume of 30 Bx lactose. H is calculated from Figs. 6-8, 6-12 (A) and (B).
Fig. 6.17 Effect of Increasing Concentration and Feed Volume on Tailing Factor (TF). A: Curve I - varying concentration of ionic matter (CaCl₂), Curve II - varying concentration of non-ionic matter (lactose).
B: Curve III - varying feed volume of ionic matter (NaPi), Curve IV - varying feed volume of non-ionic matter (lactose).
TF is calculated from Figs. 6-8, 6-12 (A) and (B).
Fig. 6-18 Elution patterns for separation of salt and lactose with varying feed volume (A) 110 cm of resin bed length. Chromatographic conditions: same as Fig. 6-12. --- NaH₂PO₄, --- Lactose.
Fig. 6-18 Elution patterns for separation of salt and lactose with varying feed volume (B) 53 cm of resin bed length. Chromatographic conditions: same as Fig. 6-12. --- NaH₂PO₄, — Lactose.