AN EVALUATION OF FOOD GUMS FOR ENCAPSULATING 
ENZYMES TO ACCELERATE CHEESE RIPENING

Thesis submitted in fulfilment of the requirements for the degree of 
Master of Science (Honours) in Food Science and Technology

BY

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Thesis panel

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April 1997
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
DECLARATION

This is to certify that the work presented in this thesis has not been submitted to any other University or Institution for a higher degree

Henry S Lam
ACKNOWLEDGEMENTS

I would like to first of all thank the almighty God for guiding me to reach this stage of my study. My heart felt thanks also go to my sponsors, Australian Agency for International Development (AUSAID) who made this undertaking possible. This thesis was also made possible due to the support and assistance of my helpful supervisors. Lots of thanks to Dr. Kailasapathy for all the help, intellectual stimulation, enthusiasm during the research and preparation of this thesis. To my other co-supervisor, Associate Professor Jim Hourigan who at various times during this project have had a major impact. Thank you Jim for the useful discussions we have had and your ever forthcoming guidance.

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ABSTRACT

Selected food gums (hydrocolloids) were tested for their abilities to encapsulate enzymes for accelerating cheese ripening. The effect of pH and acidity on gel strength of the gums was determined. Enzyme was entrapped in κ-carrageenan, gellan gum and milk fat and incorporated into cheese milk prior to cheese making. The cheese produced was tested for protein breakdown and amino acid production during ripening and textural and sensory properties of the ripened cheese were also evaluated. The findings and significance of this study and the literature review are presented.

Gels produced from alginate, agar, α- and κ-carrageenan, gellan and κ-carrageenan-locust bean gums were treated for 24 to 48 h in solutions of 0.4, 0.8 and 1.2 % acidities and of pH 4.8, 5.5 and 6.2. Most gels showed reduced strength after treatment in solutions of either modified acidities or pH. There was, however, no significant change in gel strength between the different treatments for most of the gums. Only κ-carrageenan gels showed reduced strength when solution acidity was increased from 0.4 to 1.2 % and pH reduced from 6.2 to 4.8.

The activity of encapsulated enzyme were also investigated. Encapsulation efficiencies of the enzymes ranged from 29.1 to 56.8%. Enzymes encapsulated in κ-carrageenan, gellan and alginate gums retained higher activities than the other gums studied. Enzymes entrapped in agar gels had the least retention of activity while those entrapped in α-carrageenan and κ-carrageenan-locust bean gum showed average retention of enzyme activity.

The retention of enzyme capsules produced from gellan, κ-carrageenan and milk fat in cheese curd was investigated. Loss of encapsulated enzymes in cheese whey was also determined. The retention rate for enzymes in the cheese curd ranged between 91.5 to 73.5%. Capsules of two gums viz., κ-carrageenan and gellan gums, showed higher retention rates than that of milk fat capsules. Enzyme losses in the whey
ranged from 5.6 to 17.9% with the highest losses observed with milk fat and the least with gellan gum capsules.

Proteolysis and microbial growth was determined in ripening experimental cheeses and changes in rheological and sensory parameters were also determined in ripened experimental cheeses. After 5 months, the highest proteolysis was recorded in cheeses treated with κ-carrageenan capsules. β-Casein content of the trial cheeses were lower than that of the control cheese after 5 months. The β-casein content remaining in cheeses after 5 months ripening ranged from 22.2 to 57.3%. Trial cheeses treated with κ-carrageenan contained the lowest β-casein after 5 months maturation.

Most of the cheeses treated with enzyme capsules showed higher levels of amino acid within two weeks than the control cheese. The concentration of free amino group for the experimental cheeses ranged from 0.664 to 1.496 mg/mL as compared to 0.695 mg/mL for control cheese. After two weeks, all experimental cheeses showed higher production of amino acids than the control cheese.

The addition of enzyme capsule to cheese did lead to a higher growth level of microorganisms. After one day maturation, total microbial count in experimental cheeses ranged from 38 to 52 x 10⁶ as compared to 32 x 10⁶ for control cheese. The difference in total microbial count was however not significant (P<0.05) after one month maturation. The experimental cheese exhibited lower score than the control cheese for most textural properties. The experimental cheeses were not significantly different in flavour and aroma from the control cheese. κ-Carrageenan treated cheeses were recorded as having the highest score for bitter after taste. Except for those cheeses treated with κ-carrageenan capsules, the overall acceptability for the trial cheeses were not significantly different from that of 6 month old untreated cheese.
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<tr>
<td>AMF</td>
<td>Anhydrous milk fat</td>
</tr>
<tr>
<td>$A_w$</td>
<td>Water activity</td>
</tr>
<tr>
<td>Ba</td>
<td>Barium</td>
</tr>
<tr>
<td>Ca$_2^+$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>CFE</td>
<td>Cell free extract</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbondioxide</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>Cesium ion</td>
</tr>
<tr>
<td>DRV</td>
<td>Dehydration-rehydration vesicles</td>
</tr>
<tr>
<td>DTT</td>
<td>D, L-dithiothreitol</td>
</tr>
<tr>
<td>DVS</td>
<td>Direct vat cultures</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EE</td>
<td>Encapsulation efficiency</td>
</tr>
<tr>
<td>EEC</td>
<td>European Economic Community</td>
</tr>
<tr>
<td>EMC</td>
<td>Enzyme modified cheeses</td>
</tr>
<tr>
<td>EPM</td>
<td>Enzyme per weight of matrix</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organisation of the United Nations</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FDM</td>
<td>Fat in dry matter</td>
</tr>
<tr>
<td>FFAs</td>
<td>Free fatty acids</td>
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<tr>
<td>GDL</td>
<td>D-glucono-d-lactone</td>
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<tr>
<td>Gh</td>
<td>Cheeses treated with gellan capsules containing 2.127g enzyme</td>
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<td>GI</td>
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<td>Gm</td>
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<tr>
<td>GRAS</td>
<td>Generally recognised as safe.</td>
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<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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HCl - Hydrochloric acid
HMF - High melting fat
HPCE - High performance capillary electrophoresis
HTST - High temperature short time
Γ - Iodide ion
IDF - International Dairy Federation
K⁺ - Potassium ion
Kh - Cheeses treated with k-carrageenan capsules containing 2.035g enzyme
KI - Cheeses treated with k-carrageenan capsules containing 0.342g enzyme
Km - Apparent Michaelis constant
Km - Cheeses treated with k-carrageenan capsules containing 1.036g enzyme
LAB - Lactic acid bacteria
LAPU - Leucine Aminopeptidase unit
LMF - Low melting fat
LPL - Lipoprotein lipase
LSD - Least Significant Difference
MFGM - Milk fat globule membrane
Mg - Magnesium
Mg²⁺ - Magnesium ion
Mh - Cheeses treated with milk fat capsules containing 2.206g enzyme
Ml - Cheeses treated with milk fat capsules containing 0.359g enzyme
MLV - Multilamellar vesicles
Mm - Cheeses treated with milk fat capsules containing 1.145g enzyme
MNFS - Moisture in non fat solid
N - Nitrogen
NaCl - Sodium chloride
NAD - Nicotinamide adenine dinucleotide
NADH - Reduce nicotinamide adenine dinucleotide
NaOH - Sodium hydroxide
NH₄⁺ - Ammonium ion
NSLAB - Non starter lactic acid bacteria
P - Total protein content of capsules applied to trial cheese milk.
<table>
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<tr>
<td>PGE</td>
<td>Pregastric esterase</td>
</tr>
<tr>
<td>PTA</td>
<td>Phosphotungstic acid</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>Ribodium ion</td>
</tr>
<tr>
<td>REV</td>
<td>Reverse phase evaporation vesicles</td>
</tr>
<tr>
<td>SCN⁻</td>
<td>Isocyanide ion</td>
</tr>
<tr>
<td>SFE</td>
<td>Supercritical fluid extraction</td>
</tr>
<tr>
<td>S/M</td>
<td>Salt in moisture</td>
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<tr>
<td>Sr</td>
<td>Strontium</td>
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<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>$T_{gel}$</td>
<td>Gelation Temperature</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzenesulphonic acid</td>
</tr>
<tr>
<td>$T_o$</td>
<td>Temperature of onset of helix formation</td>
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<td>TPA</td>
<td>Texture Profile Analysis</td>
</tr>
<tr>
<td>UF</td>
<td>Ultrafiltered</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>USFDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>VLFM</td>
<td>Very low melting fraction</td>
</tr>
<tr>
<td>Vmax</td>
<td>Maximum theoretical rate of enzyme-catalysed reaction</td>
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<td>WC</td>
<td>Protein content of control cheese whey</td>
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<tr>
<td>WT</td>
<td>Protein content of trial cheese whey</td>
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<td>XPDAP</td>
<td>X-prolyldiaminopeptidyl peptidases</td>
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INTRODUCTION

Although certain cheeses are consumed fresh, most cheese varieties are consumed only after a ripening or maturation period which varies from few weeks to as long as two years. Many varieties may be consumed at any of several stages of maturity, depending on the flavour preferences of consumers and economic factors. The maturation of cheese is responsible for unique characteristics of the individual cheese such as flavour, aroma and texture (Grippon et al., 1991). These characteristics are important as most people consume cheese principally for its organoleptic qualities. However, the maturation period of cheese introduces extra costs to the production of cheese. This economic aspect associated with long cheese maturation period has in recent years drawn attention from researchers around the world. For instance, Cheddar cheese takes at least 6-12 months (even longer) before sales and Permasan up to two years (Robinson, 1995). Such long periods represent a significant cost in handling and capital. Ripening alone is said to cost about US $1.50 per ton per day (Fox, 1993b) and constitutes approximately 6-8% of the total production cost in the United Kingdom (Law, 1987). Obviously, ripening cost factor of cheese especially those of low-moisture, and slow-ripening varieties is of major concern to producers who are also faced with stiff competition prevalence in both the domestic and export markets.

In spite of the high cost associated with long period of cheese maturation, recent trends have indicated that consumer preferences have turned in favour of fuller-flavour in slow-ripening varieties such as Cheddar cheese (Anon, 1995) For such varieties, under normal practice, full flavour is attained only after their long ripening periods. This demand by consumer for fuller-flavour cheese and the adoption of new technologies in cheese production has put more strains on producers. Advances in technology and changing market has introduced new goals for cheese ripening systems with an adverse effect on cheese flavour. For instance, the introduction of the modern fast starter cultures such as direct vat cultures, DVS, are not known to give a rounded and the desired strong mature Cheddar cheese flavour. DVS starters have been adopted by many cheese producers because they are less prone to phage
attack hence lead to less vat loss unlike the original bulk starters. Some of these DVS cultures have actually resulted in bitter tastes (Bech, 1992). Similarly, certain types of cheese made from ultrafiltered (UF) milk tend to ripen more slowly than normal cheese (Law and Goodenough, 1995). In recent years, due to the increasing prevalence of diet-related diseases in the western world, consumers have increasingly demanded low fat food varieties. For the cheese market, this has meant focusing attention on methods of manufacturing low and reduced fat cheese such as Cheddar without compromising flavour and mouth feel. Consumers buy Cheddar cheese because they enjoy the flavour and mouthfeel. Most of the current low fat Cheddar type cheese available have poor mouthfeel, are bitter and are slow to mature (Fox et al., 1993).

Most cheeses are made from milk by dividing or separating the milk into curd and whey with the aid of acids, enzymes or both and heat. The curd which is the semi-solid fraction later become cheese. The curd contains most of the insoluble components of the milk along with some moisture and some soluble materials. The major constituents of the curd such as protein (casein), fat and lactose are very important substrates during cheese manufacture. They are critical in the development of the final cheese characteristics. During cheese maturation, these curd components are slowly degraded into different products which are responsible for the sensory and textural quality of cheese (Fox et al., 1995).

The protein component of the curd, mainly casein, is broken down during proteolysis with the aid of protease enzymes into free amino acids (Aston et al., 1983). The fat is degraded by esterases and lipases into FFAs (Scott, 1986). Lactose is fermented to lactic acid. The enzymatic degradation products of these major constituents of the curd are known to be responsible for the development of the final flavour and texture of mature cheeses. Thus, enzyme activities are important not only for the process of maturation but also for the final quality of the cheese.

In light of the extra production cost due to long maturation periods of certain cheeses and the poor flavour quality of cheese due to the adoption of advanced production
technology, several techniques have been investigated to reduce the maturation period and strengthen flavour. These techniques include elevated temperature, modified starters, seem-liquid slurry and enzyme addition (Law, 1984). Because the first three methods are sometimes difficult to control and have resulted into putrefaction (Hayashi, 1996), it was reported that the addition of enzymes such as beta-galactosidase (Gooda et al., 1983), lipase (Lin et al., 1987), proteinase (Alkalaf et al., 1988; Law and Wigmore, 1982a) and peptidase (Law and Wigmore, 1983) was a reliable and reproducible method.

It is widely accepted that strongly flavoured cheese can be produced in a short time by adding commercial food-grade proteinases (Lawrence et al., 1987). However, both the type of enzyme and the amount used are critical in achieving the correct balance of flavour. An excess of one flavour component can upset this balance and give cheese undesirable off-flavours. During earlier studies in this area, Law and Wigmore (1982) employed an optimum concentration (0.00125 % w/w) of a bacterial neutral proteinase derived from Bacillus subtilis (Neutrase). They obtained significant increase in small peptides and amino acids though an increase of more than 150% resulted in a bitter cheese with an excessively meaty flavour. They (Law and Wigmore, 1983) later overcame this problem by supplementing the Neutrase, which has a predominantly endopeptidase activity, with an exopeptidase enzyme. Exopeptidase which has a minimal effect on flavour intensity, acts on products of endopeptidase activity to give a high yield of low molecular weight nitrogenous compounds (El Soda, 1986).

This approach of using enzyme cocktail has also been investigated by other researchers (Braun and Olson, 1986a; Grieve, 1982; Tatematsu et al., 1982; Mucchetti et al., 1983; Skeie et al., 1995; Hayashi, 1996) with promising success. This is because, in contrast to the single enzyme application, enzyme mixtures give a balanced cheese flavour. Use of enzyme combination resulted in reduction of the total enzyme dose and caused only minimal texture defects (Law and Wigmore, 1983). Commercial enzyme cocktails for accelerating cheese ripening, Accelase (Mauri), Flavourage (CHR. Hansens), NaturAge (Miles) (Fedrick, 1987) and Flavourzyme (NOVO) are
available in the market and have been reported by their manufacturers to give a typical cheese flavour in approximately half the usual ripening time.

The acceleration of cheese ripening has been investigated using free enzymes added either directly to the milk or to the curd. However, the addition of free enzymes caused premature proteolysis leading to a loss in yield, and variations in flavour and texture of the cheese produced. It was then suggested that microencapsulation technology, which duplicates some of the protective and selective properties of natural membranes, be used to entrap enzymes for controlled and faster production of flavour compounds in cheese.

It has been shown through various studies that the use of encapsulated enzyme for accelerating cheese ripening has several advantages over the application of free enzymes. Encapsulation of enzyme or enzyme systems increases the local concentrations of both substrate and enzymes in cheese thus reducing losses in whey (Braun and Olson, 1986a). This implies very efficient use of enzymes and no contamination of whey which can then still be used for processing into other food products. Encapsulation also enhances uniform distribution of enzyme in milk and protects milk proteins against premature enzyme attack (Kirby and Law, 1987).

Several materials have been studied and used for encapsulating ripening systems, such as micro-organisms and enzymes, in cheese. Many researchers (Magee et al., 1981; Braun and Olson, 1986a,b) used milk fat to coat enzymes responsible for flavour production in cheese. This technique, however, was not successful due to the lower melting point of milk fat. The best enzyme encapsulation efficiency was only obtained with the aid of non-food grade emulsifiers. Other researchers investigated liposomes as a matrix for entrapment of cheese ripening enzymes (Alkhalaf et al., 1988; Piard et al., 1986; Kirby et al., 1987). Several factors, however, limited large scale application of liposome entrapped enzymes. These included low encapsulation efficiency, poor liposome stability, lack of understanding of how enzymes are released from liposomes and the use of organic solvents during liposome preparation (El Soda et al., 1989).
Gums, especially those that form gels, could provide very good enzyme entrapment matrices. Gums are non-toxic, suitable for simple encapsulation technique and allows high viability and productivity of the entrapped enzymes or cells (Audet and Lacroix, 1989). Gums are also biocompatible and acceptable in food applications. Acceptability arises due to the tendency in the food industry to move towards the use of natural and non-toxic ingredients in foods.

Entrapment in polymeric gels is a very popular immobilisation method and has been favoured by numerous workers (Godia et al., 1987; Klein et al., 1985). As the name implies, it is merely the trapping of enzymes or cells within three-dimensional matrix of a polymer. Various types of polymers have been used for gel entrapment. Mechanisms for gel formation are varied and includes ionotropic, thermal, precipitation, and polymerisation (Willaert and Baron, 1996).

There are however no published comprehensive studies on the use of food gums for encapsulating enzymes for accelerating cheese ripening. Consequently, there is hardly any published information on the ability of food gums to release the encapsulated enzymes at controlled time of the cheese production. Therefore, the formation of and characteristics of gum gels need to be examined with respect to their suitability for entrapping enzymes. It is also important to determine the ability of the gum gels to release the entrapped enzymes under the physical and chemical conditions of cheese manufacture.

It is the aim of this thesis, therefore, to determine the feasibility of using food gums for encapsulating enzymes for accelerating cheese ripening by studying the behaviour of certain food gums such as agar, alginate, gellan, kappa- and iota-carrageenan and kappa-carrageenan-locust bean gum under cheese making conditions. These gums were chosen for evaluation because they have been frequently used in the past for entrapment purposes especially the entrapment of living cells destined for food application. Some have also been tested in sustained release formulations in pharmaceutical studies (Alhaique et al., 1996). These gums also gel at relatively mild temperatures, a factor very important for maintaining encapsulated enzyme activity.
Two gums, gellan and kappa-carrageenan, which showed better encapsulation properties than the other gums were used together with hard milk-fat fraction to encapsulate the enzymes. The encapsulated enzymes were applied to cheese milk at three levels of enzyme strength and there was also control cheese (no encapsulated enzymes applied). The resulting cheeses were then tested for proteolysis, microbial growth, textural change, organoleptic and sensory attributes and compared to a control cheese.

Cheese manufacture is a time-tested skill and involves many intricate and sometimes less well understood biochemical processes which need to be considered when contemplating any alteration to any stage of its traditional process. To appreciate this, a brief review of the historical background to cheese making and biochemistry of cheese ripening follows. Methods that have so far been investigated for accelerating cheese ripening including those involving food gums and milk-fat are also described and critically evaluated.
CHAPTER 1

LITERATURE REVIEW

1. HISTORICAL AND GENERAL BACKGROUND

Cheese is the common name used for a class of cultured fermented products produced throughout the world in more than 500 varieties (IDF, 1982). Cheese can simply be defined as a product made by the coagulation of milk by enzymes and/or acid with some of the whey expressed from the finished curd (Fox, 1987). Cheese is probably one of the oldest method of food preservation in the history of mankind dating from the 6000 - 7000 BC. It is made from the milk of all species of domesticated dairy animals (Holsinger et al., 1995), however, the greater proportion of total world production come from cows milk.

The origin of cheese making and its history has been well reviewed (Fox, 1993a; Scott, 1986). Crude forms of cheese were probably derived almost as soon as human domesticated animals, but the earliest records indicate that cheese originated some 8000 years ago in the ‘Fertile Crescent’ between the Tigris and Euprates in Iraq. Cheese making skills initially spread from its cradle land with the spread of civilisation in the Middle East, Greece and Rome. Later on, colonisation and population migration spread cheese making to the rest of the world.

It is not clearly documented as to when enzymes were first deliberately added to milk for cheese making. No wonder even the beginning of cheese making is left to legends. It is thought to have been discovered accidentally by a desert herdsman when milk in a bag made of a young animal stomach curdled in the hot sun. The milk must have curdled because the bag still contained the digestive enzyme rennin (Tamime, 1993). Rennin was then regarded as important for cheese making and has since 1873 been sold in small quantities to domestic cheese makers (Madden, 1995). It was crudely extracted in sodium chloride solution from the fourth stomach (abomasum) of a
newly slaughtered milk-fed calf, kid or lamb (Scott, 1986). The first industrial production of calf rennet was reported to have been by a Danish chemist, Christian Hansen, in 1874 (Madden, 1995). Extracts from certain plants, which possess milk-clotting properties, have also been in use for centuries (Robinson, 1995) to make vegetarian cheeses. These plants included lady's bedstraw, Galium verum, or butterwort, Pinguicula vulgaris (Madden, 1995).

Just like in the case of rennin, the literature is not specific about the first intentional use of enzymes to accelerate cheese ripening. One literature (Roux and Abott, 1962) cited the incorporation of lipolytic enzymes (lipases) in cheese during the manufacture of Cedara cheese.

Variants of the original primitive system of cheese production evolved with time. Different conditions of manufacturing and maturation dramatically change the conditions of the basic curd and as a result, it is possible to identify a number of basic types of cheese (Marshal, 1987).

World wide production of all types of cheese was about $14.9 \times 10^6$ tonnes in 1994 and has been increasing annually at a rate of about 4% (FAO Monthly Digest of Statistics, 1995). Today, cheese is primarily a product of European countries with a production of more than $7.0 \times 10^6$ tonnes, and those countries populated by European emigrants. Cheese is of relatively little importance in Asia, Africa and Latin America where diets are more strongly based on plant products and where no tradition of dairying exists (Holsinger et al., 1995). In Australia, cheese making used 37% of all manufacturing milk in 1993/1994 to produce 225,000 tonnes of cheese. About 60% of the Australian cheese is sold on the domestic market while half of the exported cheese goes to Japan (Anon, 1995). Of the over 40 varieties of cheeses manufactured in Australia, the traditional variety, Cheddar cheese, represents the largest quantity, more than 60%.
1.1 Cheese Manufacture

During cheese making, milk is concentrated by 6 to 12 folds, depending on the cheese variety by removing whey which contains most of the water, lactose, some minerals and some proteins. The objectives of cheese making are two folds: (1) develop the basic structure of the cheese, and (2) obtain the cheese composition required for optimum microbial and enzyme activity during curing. Cheese composition here refers to optimum levels of moisture, fat, pH (lactic acid), minerals (especially calcium), and salt (Hill, 1995) for a given variety.

Majority of cheese varieties have two well defined stages of production, manufacture and ripening. The manufacturing phase normally occurs within the first 24 h although operations such as salting and dehydration may sometimes take longer. Manufacturing protocols for individual varieties differ but the basic steps common to most varieties are acidification, coagulation, dehydration, and salting. Acidification may either be brought about by the addition of acid or by the activities of starter bacteria. Starter bacteria basically is for lactic acid production and is added to vat milk to give \(\sim 10^6\) to \(10^7\) colony-forming units (cfu)/ml. This amount and type depends on the cheese type and characteristics desired. Lactic acid is responsible for the fresh acidic flavour of unripened cheese and is important for milk coagulation. Coagulation of casein into a curd is accomplished by the activity of rennet (limited proteolysis). The coagulated curd, which is immersed in whey, forms a gel that entraps most fat present. Most of the whey is separated from the curd during the dehydration process. Dehydration involves the post-coagulation treatments that break/cut the gel, resulting in expulsion of whey.

The curds for most ripened cheeses are cooked in whey to 37-41°C depending on the variety. Cooking helps expel more whey from the curd. The whey is then drained and the curd separated. The curd is then salted and pressed overnight. Pressing further removes more whey and gives shape to the mature cheese. Salting play many roles: controlling microbial growth and activity, reducing water activity (\(A_w\)), controlling rates of \(\alpha\)- and \(\beta\)-casein breakdown, regulates formation of physical changes in
cheese protein, preventing growth of starter bacteria and causing the uncoupling of lactose fermentation. An overnight pH change of 4.95-5.30 occurs in most varieties coupled with a shift in the population of starter organisms to lactobacilli and/or pediococci (Fox, 1993a). The curd is normally packaged in a polythene film or coated in a film before storing at low temperature (8-12°C) to begin the process of ripening.

The manufacturing phases thus determine the nature and quality of the finished cheese. The next phase (ripening) is then responsible for the characteristic flavour and texture of the individual cheese varieties.

1.2 Cheese Ripening

Cheese ripening is the gradual enzymatic modification of the lactose, milk fat and protein under controlled conditions of time, temperature, and humidity which leads to the progressive development of desired body, flavour and texture of mature cheese (Gripin et al., 1991) Fig 1.1. Wide variety of cheese present in the market owe their differences in appearances and organoleptic characteristics to the various techniques of curd preparation and differences in ripening microflora. Bacterial cultures produce acid in most cheese which is essential for the development of the basic structure and composition as well as cheese flavour and texture. Each method of curd preparation imparts a unique physico-chemical condition (pH, water content, salt content) responsible for differences in the texture and flavour of mature cheeses (Fox, 1993 a).
Figure 1.1  Ripening of hard cheese, general scheme of substrate conversions leading to flavour and aroma compounds.
(Adapted from Law and Goodenough, 1995).
1.2.1 Texture

Cheese texture is very important because it is the property by which the consumer first identifies and judges a specific variety. Different workers have defined cheese texture in very different ways. Cheese texture may be defined as the primary sensory properties of cheese that relates to body, structure, firmness, hardness, consistency, cohesiveness and crumbliness. Most workers however use firmness to measure texture.

Lawrence et al (1987) in their study on cheese texture found that, cheese texture at any stage of cheese ripening depend primarily on its pH and the ratio of intact casein to moisture. This confirmed what De Jong (1976) had earlier on discovered. De Jong found a good correlation between cheese firmness and its content of intact α\textsubscript{S1}-casein. When caseins break down, the water soluble products do not make any contribution to the cheese matrix (Walstra and van Vliet, 1982). Therefore as more caseins are degraded the firmness of the cheese matrix drops.

Cheese texture is also influenced by the cleavage of peptide bonds during proteolysis (Creamer and Olson, 1982). This is because when a peptide bond is cleaved, it generates two new ionic groups each of which will compete for the available water in the system. Thus, the water previously available for solvation of the protein chains get tied up to the new ionic group. The implication for low-moisture cheese, such as Cheddar cheese, is that it tends to harden with age and become more resistant to any structural deformation (Creamer and Olson, 1982; Stanley and Emmons, 1977).

Texture development during ripening occur in two distinct phases (Lawrence et al., 1987). Phase one occurs in the first 7 to 14 days when the casein network which make up the microstructure is weakened. This is due to the hydrolysis of α\textsubscript{S1}-casein by the coagulant to give a peptide α\textsubscript{S1}-1 (Creamer and Olson, 1982). This leads to a rapid conversion of the rubbery texture of young cheese curd into a smoother, more homogenous product.
The second phase is a much more gradual change over the following months as the rest of the $\alpha_S$-casein and other caseins are broken down. This phase is determined by the rate of proteolysis and rise in pH (Lawrence et al., 1987).

1.2.2 Flavour and Aroma

It is not often easy to distinguish between the aroma and flavour of cheese because of the influence they have on each other. Both cheese aroma and flavour are influenced by cheese texture because texture affects consumer perception and release of sapid and aromatic compounds from the cheese mass during mastication (McGugan et al., 1979).

Cheese aroma arises mainly from the volatile components such as esters, fatty acids, aldehydes, ketones, alcohols, amines, hydrogen sulphide and ammonia, released during curd ripening (Scott, 1986). These aroma compounds are present in exceedingly small quantities measured in parts per million. Sulphur-amino acids, cysteine, cystine and methionine release some of these aroma compounds upon decomposing. These may impart a typical cheese aroma (acetic acid) bitter flavour (ammonia salts) or bread-like taste (methional) to cheese (Adda, 1988).

Cheese flavour is a very important quality attribute which has attracted intensive investigations by scientists since the turn of the century (Fox et al., 1995). The flavour of cheese originates from a complex series of microbial, enzymatic and chemical transformations, the relative importance of which is not yet fully understood (Braun and Olson, 1986). It was initially thought that cheese flavour might be due to a single compound but the "Component Balance Theory" (Mulder, 1952) proposed that cheese flavour is due to the correct balance and concentration of a wide range of sapid and aromatic compounds. Over 300 different volatile and non-volatile compounds are known to contribute to cheese flavour (Kristoffersen, 1973).

There are three major substrates in cheese responsible for cheese flavour: lactose, proteins and fats (Fox et al., 1995). Fermentation of lactose (and citrate in some
cases) occurs first. It takes place in the cheese vat and gives cheese curd a characteristic mild, creamy flavour (Fox et al., 1995). Research evidence over the years have suggested that the secondary metabolism of carbohydrates in cheese does not have a major significance in basic cheddar flavour. It can however bring flavour notes that may be exploitable in specific cases (Law, 1984).

The role of fat in cheese is for the perception and development of flavour. This has been proven by the fact that Cheddar cheese made from low-fat milk developed no typical flavour even after 12 months of maturation. Much of the milk lipase is destroyed during pasteurisation (72 °C/15 s), but the starter and lactobacilli esterase and lipases act on mono- and diglyceride fractions to liberate FFAs. FFAs are transformed to methyl ketones, secondary alcohol, lactones and esters. Generally, the synthesis of esters and the formation of methyl ketones are the most important transformations of FFAs in cheese ripening. FFAs are important in the flavour of many surface-ripened cheeses such as blue veined and white mould ripened cheese (Scott, 1986) where flavour is dominated by methyl ketones and FFA.

When too high an amount of short chain FFA has been produced, the undesirable rancid flavour results. Rancidity is normally the result of a high level of lipase activity. This can be observed in various types of cheese made from milk contaminated with psychrophilic microflora. It also occurs when lipase has been added for the purpose of accelerating ripening as observed in Cheddar (Law and Wigmore, 1985). Rancidity is not bad for every cheese. It is considered natural and acceptable in certain strongly piquant Italian cheeses such as Romano, Provolone and Fontina (Kilara, 1985; Sood and Kosikowski, 1979). FFA may also negatively influence cheese flavour indirectly. High FFA level inhibits certain micro-organisms responsible for the biosynthesis of other important cheese flavour components (Adda, 1986).

There is strong support for the view that the products of protein hydrolysis, especially amino acids, play an essential role in the development of cheese flavour (Aston et al., 1983). Degradation of proteins results in the formation of important non-volatile and volatile compounds (Kristoffersen, 1985). Many free amino acids are catabolized by
decarboxylation, deamination, transamination, and desulfurylation to produce amines, ammonia, acids, keto acids, carbonyls, alcohols, other amino acids, hydrogen sulphide, dimethyl sulphide, methanethiol, thio esters, and other sulphur compounds. The secondary flora generally play an important role in the catabolism of amino acids and contribute to the characteristic flavour of a given cheese variety. The principal contributors to aroma are less clear but sulphur-containing compounds, especially methanethiol, and carbonyl compounds are major contributors to the flavour of Cheddar and other varieties (Kristoffersen, 1985; Manning et al., 1984,). This knowledge is the justification for the several efforts made by researchers to generate typical cheese flavours from milk proteins (Law, 1980; Law, 1983).

Flavour is not only derived from the volatile compounds of proteolysis but also emanate due to interactions between certain compounds (Biede and Hammond, 1979a,b). The typical sweet flavour of Swiss cheese for instance arises from an interaction between calcium and magnesium with peptides; the brothy-nutty flavour is with small peptides and amino acids, meanwhile the burned-bitter flavour is due to medium sized (tri-, hexa-) peptides.

Protein also causes the flavour defect, bitterness, in different cheese varieties (Mohammad and Lee 1996). In cheese, bitterness results from the presence of low molecular weight hydrophobic peptides arising mainly from casein breakdown. The \( \alpha_{S1} \)-casein produces more bitterness than \( \beta \)-casein (Rank et al., 1985). This may explain why goat and ewe whose milks contain relatively little \( \alpha_{S1} \)-casein are devoid of any bitterness (Adda, 1986).

Several factors (Mohammad and Lee, 1996) such as manufacturing conditions, rennet, but above all, cheese microflora, have been shown to influence the formation of bitter peptides. But overall, bitter peptides only remain in cheese because of the inability of peptidase-deficient starter strains to degrade them to non-bitter peptides and amino acids (Stadhouders and Hup, 1975). In mould ripened cheese however, the starter \textit{Pencillium caseicolum} itself causes bitterness if its growth is significant (Vassal and Grippon, 1984).
Bitterness in cheese rose into prominence with the use of bacterial or fungal proteinases (Sood and Kosikowski, 1979) to accelerate cheese ripening. Excessive bitterness resulted from those experiments (Law, 1983) due to imbalance hydrolyzation of milk caseins. Since then, several techniques have been used to control bitterness in cheese. The most popular one being the combined application of exopeptidases and proteinases such as the one used in this study.

As for the starter bacteria, they produce the substrates, such as amino acids, and the favourable conditions (pH, Eh) which favour the conversion of these substrates to the compounds responsible for the final flavour (Law, 1984).

1.3 Enzymes in Cheese Ripening

Cheese ripening corresponds to the enzymatic modification of caseins, fat and carbohydrates found in the fresh curd (Grippon et al., 1991). According to Abrahamson et al (1989), the enzymes responsible for this transformation originate from: milk itself (endogenous enzymes), milk microbial flora, additives for cheese making (coagulating enzymes), starter-organisms or other cultures of microorganisms added to the milk or to the cheese.

1.3.1 Endogenous Milk Enzymes

Bovine milk is reported to have some 60 indigenous enzymes originating from the mammary gland tissue cells, blood plasma and blood leucocytes (Kitchen, 1985). Many of them have the potential to contribute to various aspects of cheese ripening. However, most of them have no known technical significance in milk because they are destroyed during high temperature short time (HTST) pasteurisation of milk (Scott, 1986) and have very low activity.

Those indigenous milk enzymes most likely to contribute to cheese ripening are plasmin, lipoprotein lipase, acid phosphatase and xanthine oxidase. Lactoperoxidase can significantly contribute towards cheese quality through the activation of their antibacterial activity. Lactoperoxidase can be activated by addition, or in-situ
production of $\text{H}_2\text{O}_2$ and perhaps SCN$^-$. The role of phosphatase, especially the heat stable acid phosphatase, in cheese ripening is questionable. Most researchers believe it participates in the dephosphorylation of peptides derived from casein degradation. Xanthine oxidase reduces nitrate to nitrite in cheese thus inhibiting the germination of *Clostridium tyrobutyricum* spores. *C. tyrobutyricum* may cause the undesirable late bloating of cheese. All these enzymes, with the exception of lipase, are fairly stable and survive HTST pasteurisation almost unaffected. Hence can still be important in pasteurised milk cheese. Lipase may also remain important because some of their activity have been detected in pasteurised milk (Fox and Stepaniak, 1993).

### 1.3.1.1 Lipases

Milk contains two endogenous lipases, bile salt-stimulated lipase and lipoprotein lipase (LPL). Unlike LPL which is found in all mammals, the former is not present in milks of domesticated animals but present in human milk. LPL is a well-characterised lipase which belongs to a member of lipases family which catalyse steps in the digestion and transport of triglycerides (Olivecrona et al., 1992). The main source of LPL in the mammary gland is assumed to be the milk-producing alveolar epithelial cells (Camps et al., 1990). They appear to transfer to casein micelles upon their assembly and released together in milk with the casein micelles. Transfer to the milk fat globules occur later during milk collection and cold storage. LPL may also leak out of the mammary gland because of tissue damage for instance during cases of mastitis.

Lipoprotein lipase hydrolyses the 1,3-position in tri-, di- and monoglycerides and the 1-position in glycerophospholipids. It does not hydrolyse cholesterol esters or sphingolipids at significant rates. LPL preferentially produces short and medium chain FFA during hydrolysis of milk triglycerides. The FFAs may produce an unpalatable rancid or 'lipolysed' flavour. In bovine milk, more than 80% of the LPL is associated with the casein micelles (Olivecrona et al., 1992) and is presumably incorporated into cheese. LPL probably causes significant lipolysis in raw milk cheese and it may also contribute to lipolysis in pasteurised milk cheese since heating to
≥ 78 °C for 10 s may be required for its complete inactivation. This heating is far higher than the normal HTST (72 °C for 15 s).

It is not known how much contribution, if at all, lipolysis makes towards the ripening of Cheddar, Dutch and Swiss cheese varieties, where, even moderate lipolysis is undesirable. Lipolysis could however be important in cheeses of the Italian varieties where rancid flavours are acceptable (Scott, 1986).

1.3.1.2 Proteinases

There are two main indigenous proteinase systems in milk; neutral-alkaline proteinase and acid proteinase. Milk proteinases are important because they partially survive HTST pasteurisation of milk (Reimerdes et al., 1979). The main enzyme in the neutral-alkaline system is a typical serine protease with trypsin-like activity called plasmin (Halpaap et al., 1977). Most (~90%) of the plasmin in bovine milk exists as its precursor, plasminogen. Both plasmin and plasminogen are associated with casein micelles in milk (Korycka-Dahl et al., 1983) and are thus concentrated in cheese (Farkye and Fox, 1990). Plasmin and plasminogen in milk appears to be derived from the blood (Grufferty and Fox, 1988). This view is supported, albeit indirectly, by the fact that their activities increase during mastitis. Plasmin activity also increases by pasteurisation or cooking cheese curd at temperatures > 50 °C (Farkye and Fox, 1990). The pasteurisation and cooking temperatures inactivate the inhibitors of plasminogen activator (Richardson, 1983).

Plasmin attacks all types of caseins especially $\alpha_{S2}$-, and $\beta$-caseins in the milk (Grufferty and Fox, 1988), breaks down $\beta$-casein to produce $\gamma$-caseins (Eigel, 1977) and part of the proteose peptone fraction (Andrews and Alichanidis, 1983). $\alpha_{S2}$-Casein is rapidly hydrolysed by plasmin to several peptides. Plasmin and their activities have been identified in various cheese types such as Swiss, Cheddar (Richardson and Pearce, 1981), Gouda (Creamer, 1974) and Romano (Guinee and Fox, 1984) during ripening. Farkye and Fox (1991) found that plasmin contributes significantly to the formation of water soluble nitrogen in Cheddar. Plasmin activity
was, however, found to be higher in Swiss and Romano, probably due to their higher scalding temperature (Farkye and Fox, 1990). These workers, Farkye and Fox (1992), later successfully accelerated cheese ripening by applying exogenous plasmin in cheese milk.

The acid proteinase, is thought to be the lysosomal acid proteinase, cathepsin D, (Kaminogawa et al., 1980) and was confirmed recently when its zymogen, procathepsin D, was identified in bovine milk (Larsen et al., 1993). It has a similar proteolytic activity characteristic as chymosin (McSweeney et al., 1994). Acid proteinase hydrolyses α₄₁-casein more rapidly than β-casein. In milk acid proteinase is associated with casein and is thought to be active during cheese making.

These findings provide support to some earlier suggestions that natural milk proteinases are involved in the formation of amino acids during Cheddar cheese ripening (Visser, 1977).

1.3.2 Exogenous Enzymes

One of the earliest applications of exogenous enzymes in the food industry, dating back to about 6000 BC, was the use of rennet in cheese manufacture (Fox, 1993a). Traditionally, rennets were prepared from calves, kids or lambs stomachs and the principle proteinase in such rennets is chymosin (Foltmann, 1987). Due to the ever rising world-wide production of cheese and the concomitant fall in calf rennet supply, microbial proteases from Mucor or Endothia parasitica have been used as substitute for animal rennet. However, microbiological rennin has been found to be less specific than pure chymosin (Wasserman et al., 1988). Pepsin from the stomach of adult cattle has also been used.

Rennet is used in the manufacture of most ripened and some fresh cheeses. It coagulates milk in two stages. In the first stage, it cleaves κ-casein at the Phe 105-Met 106 releasing para-κ-casein (κ-Casein f1-105) and an hydrophilic peptide (κ-CN f106-169). This destabilises the casein micelles. The hydrophilic peptide, macropedptide, is lost into the serum or whey. These events lead to the second stage which is the
aggregation of the casein micelles. This occurs due to the reduction of the surface charges on the \( \kappa \)-casein upon hydrolysis (Dalglish, 1984). The aggregation rate of renneted micelles is unaffected by the rennet concentration or by the size of the micelles. It is however very sensitive both to the concentration of calcium ions present in the solution and to the temperature (Dalglish, 1983).

Depending on the rennet type, pH, and cooking temperature, only about 6% of the rennet added to cheese milk is normally retained in the curd (Fox, 1993a). The residual chymosin degrades both \( \alpha_{S1} \)-casein and \( \beta \)-caseins (Fox, 1989; Fox et al., 1993). \( \beta \)-casein in solution is sequentially hydrolysed to yield peptides but its hydrolysis by chymosin in bacterially-ripened cheeses is not significant (Fox, 1993a). Microbial rennet however cause extensive hydrolysis of \( \beta \)-casein in cheese.

\( \alpha_{S1} \)-Casein is very easily hydrolysed by chymosin (McSweeney et al., 1993). It is completely degraded to f1-23 and f24-199 (\( \alpha_{S1-1} \)) peptides in Cheddar and Dutch-type cheeses. This hydrolysis is dependent on pH and NaCl concentration (Fox, 1993a) and is responsible for the softening of cheese texture during early ripening (De Jong, 1977). The hydrolytic action of chymosin also influences cheese flavour in three ways (Fox, 1993b):

Rennet-produced peptides may negatively or positively influence flavour directly depending on the extent of proteolysis or the physico-chemical conditions (high moisture or low NaCl results in bitterness).

Chymosin produce peptides that act as substrates for microbial proteinases and peptidases. The microbial enzymes initially release small peptides and amino acids that contribute to either the background flavour or even to bitterness and later the amino acids are further degraded to sapid compounds, amines, acids, NH\(_3\), thiols, responsible for typical cheese flavours.

- changes in cheese texture influence the release of flavourful and aromatic compounds of proteolysis, lipolysis, glycolysis and secondary metabolic changes during mastication.
Good quality rennet extracts are free of lipolytic activity but the rennet paste used in the manufacture of some Italian varieties (e.g., Romano, Provolone) contains a potent lipase, pregastric esterase (PGE), which catalyses the extensive lipolysis responsible for the "picante" flavour characteristic of such varieties (Nelson et al., 1977). Rennet paste is made from dried, ground and slurried abomasum of calves, kids or lambs slaughtered immediately after suckling.

1.3.3 Enzymes from Starter Cultures

Starter cultures are micro-organisms, lactic acid bacteria (LAB), used during fermentation of dairy products to produce lactic acid. They fall under two categories: thermophilic with an optimum temperature of around 45°C or mesophilic with an optimum temperature of around 30°C. Each category has different species of LAB (Cogan and Accolas, 1990). Thermophilic cultures include *Streptococcus thermophilus* and *Lactobacillus helveticus* and/or *Lac. lactis*. Mesophilic cultures contain *Str. cremoris* and *Str. lactis* but aroma and flavour producers such as *Str. diacetylactis* and *Leuconostoc spp.* may also be found.

Lactic acid is important for the distinctive and fresh, acidic flavour of fermented milk. In cheese making, lactic acid coagulates milk and texturises the curd (Tamime, 1990). The cheese curd produced is gradually digested by enzymes during ripening. One major source of the cheese ripening enzymes is the LAB itself (Chapman and Sharpe, 1990). Starter bacteria may release proteinases extracellularly into the cheese matrix during growth (Exterkate, 1979) or when they are degraded by their own hydrolytic enzymes (Thomas, 1987) upon cessation of growth.

It has since been recognised that there exists a weak lipolytic activity in *Lactococcus* spp. This contributes significantly to lipolysis in the Cheddar and Dutch cheese varieties where the strongly lipolytic surface flora are absent. Fryer et al. (1967) indicated that the weakly lipolytic lactococci will hydrolyse milk fat to a significant extent if present in high numbers for long periods (e.g., during cheese ripening). The importance of starter lipase was proven in a study which showed no accumulation of
FFA during an aseptic cheese ripening when starter bacteria was replaced with gluconic acid-d-lactone (Reiter et al, 1967). Tributyrin was found to be the most important substrate for the lipase system of LAB (Singh et al., 1973).

The starter is regarded as a major source of proteinases and peptidases in cheese. The proteolytic enzymes of the starter cells are released into the cheese matrix when the cells lyse after death (Fox, 1989). Starter proteinases/peptidases act primarily on large and intermediate-sized peptides produced by chymosin and plasmin, producing a range of short peptides and amino acids (O'Keefe et al., 1978). Thus the action of starter proteinases/peptidases is vital for the formation of sapid flavour compounds from casein. Lactococcal proteinase are capable of hydrolysing intact caseins in solution too, especially β-casein, with a few strains also able to hydrolyse α_{S1}-casein (Visser et al., 1986). The latter, however is not important since α_{S1}-casein is rapidly hydrolysed by chymosin and other rennets.

1.3.4 Enzymes of Milk Flora and Non-starter Bacteria.

The non-starter bacterial population in Cheddar cheese has long been known to be composed mainly of lactobacilli (Johns and Cole, 1959). The NSLAB is predominantly *Lactobacillus plantarum, L. Casei, L. Brevis*, and to a lesser extent, species of pediococci (Turner and Thomas, 1980) and micrococci (Bhowmik and Marth, 1990).

The flora of the pasteurised cheese milk consists of the organisms that survive pasteurisation (thermoduric organisms); corynebacteria, micrococci, enterococci, and spores of *Bacillus* and *Clostridium*. There are also post-pasteurisation contaminants which are derived mainly from the factory environment and personnel. These include micrococci, occasional coagulase-positive staphylococci, coliforms, and LAB (Muir, 1990).

Cheddar cheese contain small quantities of lactate and citrate which are metabolised by NSLAB to produce acetate and diacetyl (Thomas, 1987) respectively. Both acetate
and diacetyl are regarded as significant contributors to Cheddar cheese flavour (Scarpellino and Kosikowski, 1962). Pediococci and Micrococci both produce lipase and are possible contributors to lipolysis during ripening (Bhowmik and Marth, 1990). Another important source of potent lipase in cheese are the psychrotrophs, when present in refrigerated cheese milk at numbers above 10⁷/ml (Cousin et al., 1977). These lipases are important in cheese for two reasons: many psychrotroph lipases are heat stable and, psychrotroph lipases adsorb onto the fat globules and get concentrated in the cheese unlike their proteinases counterparts which are lost in whey. This may cause rancidity in ripening cheese.

A wide range of proteolytic enzymes have been identified in NSLAB which make them likely to contribute to proteolysis in cheese. Most investigations have been done on the proteolytic systems of lactococci rather than lactobacilli (Khalid and Marth, 1990a). Several peptidases, such as intracellular peptidases, dipeptidases, aminopeptidases and endopeptidases, have been found in Lactobacillus.

Strains of L. plantarum and L. casei preferentially degrade β-casein and less α-casein (Khalid and Marth, 1990b). L. casei also has carboxypeptidase activity which is absent in lactococci (El Soda et al., 1978). Previous studies (Bhowmik and Marth, 1988) indicated that Micrococcus spp. produce both intra- and extracellular proteinases. The proteinases were observed to hydrolyse whole casein, αS₁- and β-caseins (Garcia de Fernando and Fox, 1991). Proteolytic activities of Pediococcus have not been well documented, nevertheless, El Soda et al (1991) reported the presence of aminopeptidase and dipeptidase activities in Pediococcus.

1.4 Accelerating Cheese Ripening

Cheese manufacture aims at the preservation of principal nutrients in milk in a safe, and palatable form. Equally important is that a certain time lag is required between the initial milk conversion and the realisation of flavourful cheese with right aroma and texture. Such time lag has several disadvantages associated with it as indicated at the introduction section of this thesis.
Having acquired a general understanding of the biochemical processes that occur during cheese ripening, researchers have attempted to apply this knowledge toward speeding up the reactions which generate flavour and modify texture. The aim of accelerating the various biochemical pathways is to reduce the ripening time without adversely affecting flavour and texture. Approaches that have been and/or are currently under investigation for accelerating the ripening of cheese can be categorised into four main groups (Fox, 1993b): elevation of temperature, modified starters, exogenous enzymes and cheese slurries.

1.4.1 Elevated Ripening Temperature

A number of cheese varieties are ripened at low temperatures, for instance Cheddar is ripened at 6-8°C (Fox, 1988). But most chemical and enzymatic reactions are known to take place at a higher rate when the reaction temperature is raised. Consequently, the biochemical reactions that lead to the production of sapid and flavour compounds during cheese maturation should occur faster at higher temperatures. One of the first attempts to use a higher temperature to reduce the maturation time of cheese was made by Sanders et al (1946) as quoted in El Soda (1993). They showed that Cheddar cheese made from pasteurised milk and matured at 16°C was fully matured in 3-4 months without defects in flavour and quality. Later Law et al (1979) while investigating the influence of various factors on flavour intensity of Cheddar cheese found that ripening temperature was the single most important factor. Cheese ripened at 13°C scored higher for flavour intensity than those ripened at 6 °C where bitterness was also more marked.

In other studies, Aston et al (1983) and Fedrick and Dulley (1984) assessed the influence of various ripening temperature regimes on proteolysis and flavour development in Cheddar cheese. They showed that the development of cheese flavour correlated well with the formation of 5% PTA soluble-N, composed basically of free amino acids and small peptides. Cheeses ripened at elevated temperature in the presence of mutant starter had higher ratio of free amino acids to peptides either due to accelerated production of peptide substrates for the starter peptidases or increased
peptidolysis. Similarly, the production of volatile sulphur compounds, hydrogen sulphide and carbonyl sulphide increased with elevation of ripening temperature. On cheese quality, cheeses became less springy and more fracturable with increasing storage temperature and time. Cheese softening which occurs initially with ripening time decreases markedly with age.

Generally, raising ripening temperature from 5-8°C to 13-15°C accelerates cheese ripening without any significant development of off-flavour as long as the cheese was of high microbiological quality and good composition prior to increasing the temperature (El Soda, 1993). This method is technologically very simple and is unrestricted by legal barriers (Wilkinson, 1990). It however suffers from the increased risk of microbial spoilage especially when the milk is of low microbial quality.

1.4.2 Use of Modified Starter Cultures

The enzymes of starter and non-starter bacteria play a very important role in the production of flavours of several cheese varieties. Starters can either be modified or attenuated to reduce their ability to produce acidity during cheese manufacture. Their proteolytic activities however remains intact. Thus applying them during cheese manufacture is equivalent to increasing the content of proteolytic enzymes without affecting acid production. This will promote more proteolysis during cheese ripening hence fasten cheese maturation without off-setting any chemical balance which may lead to undesirable product.

Although it is known that starter bacteria have proteolytic and autolytic activities, these facts have never been considered as important during their selection. Instead selection has been based on their ability to produce acid at consistent rate during manufacture. Strains such as L. lactis subsp. cremories lyse early during ripening to release the intracellular enzymes responsible for the production of flavourful cheese of good texture (Fox, 1988; Law, 1990).
The selection and use of starter strains with enhanced autolytic properties and increased peptidase activity was envisaged to excel over the use of exogenous enzymes in accelerating cheese ripening. This is because the former method provides a more balanced enzyme complement which is likely to give a cleaner and more consistent cheese flavour. The method however remains technologically very complex and most likely uneconomical.

Techniques which have been used to manipulate these cultures in order to improve their ability to accelerate cheese ripening include stimulating cells; lysozyme treatment; heat or freeze-shocking cells; spray drying and; mutating cultures.

**Stimulating Starter Cells:** By the addition of enzymes and/or hydrolysed starter cells to cheese milk promotes the growth of starter cells as seen in certain Russian cheeses (Law, 1990). Ripening of these cheeses was accelerated by using starters which were initially grown in media containing protein hydrolysates at higher temperatures. A commercial grade metalloproteinase, Rulactine, derived from *Micrococcus caseolyticus* added to milk destined for producing Emmental, Gouda and Carre de l’est cheeses resulted in faster maturation of these cheeses (Vassal *et al.*, 1982). No bitter flavour was detected. Rulactine not only contributed directly to proteolysis but most importantly produced peptides and amino acids that stimulated growth of starter and secondary flora responsible for most of the proteolysis during ripening. Similarly, Egyptian Ras cheese ripening has been accelerated by use of hydrolysates of casein or whey and trace elements (Wilkinson, 1990).

**Lysozyme Treatment:** The lysis of starter bacteria in cheese is thought to be important for proteolysis. Law *et al* (1976) in attempting to accelerate the ripening of Cheddar cheese added lysozyme-sensitised cells to cheese milk. The cells lysed and released intracellular dipeptidase which led to a significant increase in the level of free amino acids in a six month old cheese as compared to a control. But flavour remained unaffected which led them to believe that intracellular enzymes played no direct role in flavour development. The cost of lysozyme however is prohibitively
high rendering this method economically not viable at the moment for large-scale cheese manufacture (Wilkinson, 1990).

Attempts to add lysis to milk-grown cultures of \textit{L. lactis} subsp. \textit{cremoris} in the laboratory resulted in huge reduction of cell viability; only 0.04\% of cells remained viable after 72 h against 44\% for control. So far, application of lysis during cheese making trials has not been documented (Wilkinson, 1990).

**Heat- or Freeze-Shocked Treatments:** Swedish researchers (Bie and Sjostrom, 1975) while attempting to reduce the acid-producing ability and enhance the rate of autolysis of a mixed strain of starters or a \textit{L. helveticus} culture, subjected them to an array of heat-shock treatments. They noted that increasing the severity of heat treatment caused increased autolysis but did not result into a similar increase in proteolysis due to heat-denaturation of the proteinases. But a moderate amount of heat-treated \textit{L. helveticus} (69 °C for 20 s) in combination with untreated mixed-strain starters accelerated cheese ripening. Ripening period was reduced by half without the development of bitterness. Heat treatment probably resulted into early cell lysis which then contributed to the debittering of cheese.

Proteolysis was also increased in Gouda cheese supplemented with \textit{L. helveticus} previously heat-shocked at 70 °C for 18 s (Bartels et al., 1987a). They noted an enhanced flavour and debittering.

Different lactic bacterial strains have different optimum conditions for thermoshocking. Lactococci thermoshocked at 56.5°C for 17 s resulted in a 93-97\% reduction of acid production and only 15-30\% loss of the cell wall proteinase and aminopeptidase activities (Exterkate et al., 1987). The optimum conditions recommended for \textit{Lactobacillus} strains were 64°C for 18 s (Castaneda et al., 1990).

Cells with active proteolytic systems but diminished lactose-utilising ability have also been produced through freeze-thawing. Such cells are known to autolyse twice as fast as control thus accelerating proteolysis and flavour development without bitter note as
observed in Gouda cheese when supplemented with freeze-thaw *L. helveticus* (Bie and Sjostrom, 1975). Untreated *L. helveticus* does accelerate proteolysis but unlike the freeze-thawed cells, off-flavours were produced as well.

More recently, El-Soda *et al* (1991) used freeze shocked cells of *Lactobacillus casei* and *Pediococcus* LR to accelerate the ripening of a reduced fat cheese. They noted a significant increase in TCA soluble nitrogen in cheese containing freeze-d shocked cells. Cheese made with frozen *L. casei* and pediococci cells showed a 52% and 33% increase in proteolysis respectively after 6 months. Acid flavour was noted in both *L. casei* and pediococci cheeses and internal calcium lactate crystals formed in the pediococci treated cheese during ripening. The strong acid development and/or crystal formation may be great drawbacks to the application of this method for accelerating cheese ripening.

**Spray Drying:** This is the most recent physical procedure used to attenuate the acid-producing abilities of bacterial strains, without damaging their peptidase system. The technique was developed by Johnson and Etzel (1991) who produced cultures in a dry, stable powder form containing non-viable micro-organisms with active intracellular enzymes. There is however no results of cheese making experiments with the spray dried cultures available at this time.

**Mutant Starter Cultures:** Pioneering work with genetically-modified strains of starter culture involved lactose-negative (lac') or proteinase-negative (prt') strains (Fox, 1989; El Soda and Pandian, 1991: reviews). Lactose-negative mutants of starter strains are meant to enhance the production of peptides and free amino acids during cheese ripening without acid production during manufacture. Cheddar cheese produced from milk to which was added mutant *L. lactis* subsp. *cremoris* C2 (lac’ prt’) showed improved flavour, advanced ripening, increased proteolysis as compared to control and no bitter taste (Grieve *et al.*, 1983).

Lately, researchers have also focused on the use of peptidase-negative mutants (El Abboudi *et al.*, 1992). They have obtained strains lacking aminopeptidase or X-
prolyldiaminopeptidyl peptidases (XPDAP). El Abboudi et al (1992) added to the cheese curd freeze-shocked cells of \textit{Lb. casei} or its XPDAP-deficient-mutant strain while investigating the role of XPDAP during ripening of Cheddar cheese. They observed an increase of 60% soluble nitrogen in the experimental cheeses over the control but no difference in the amount of TCA or PTA-soluble N levels or flavour score.

Genetic techniques have also led to the expression of the neutral proteinase from \textit{Bacillus subtilis} in \textit{Lactococcus lactis} susp. \textit{lactis} (de Guchte et al, 1990). Over expression of proteinase (Bruinberg et al., 1992) and aminopeptidase (Alen-Boerrigter et al., 1991) has also been described. Cheese making experiments with these genetically produced strains have not yet been reported.

Genetically modified strains of starter culture could provide a uniform distribution of increased amounts of proteolytic activity in cheese. But this would only be useful as long as the techniques used to produce the enhanced starter characters are not prohibited. The technology involved also seems to be costly making it uneconomical.

\subsection*{1.4.3 Adjunct Cultures}

Certain micro-organisms such as the NSLAB have always been regarded to play a significant role during proteolysis and flavour production in cheese. Researchers have then thought of exploiting these facts to select certain NSLAB and add them along with the normal starter and increase the rate of casein degradation and flavour development in cheese. The selected NSLAB that is deliberately added during cheese manufacture are referred to as adjunct cultures.

Puchades et al (1989) added different \textit{Lactobacillus} spp. to Cheddar cheese milk and observed increased level of proteolysis and better flavour score for \textit{L. casei} subsp. \textit{casei}, and \textit{L. plantarum}. As is the case with other heterofermentative lactobacilli, \textit{L. brevis} scored the lowest for flavour and texture.
Low-fat Cheddar cheese exhibited increased proteolysis and enhanced flavour development after 3 months when strains of *Micrococcus* and *Pediococcus* were added. Cheese with pediococci scored better after 6 months than that of micrococci which developed off-flavours (Bhowmik et al, 1990). Addition of adjuncts may lead to unpredictable products because of the large number of micro-organisms involved.

### 1.4.4 Cheese Slurries

In this method, water, salt, trace elements and reduced glutathione are added to cheese before incubation under anaerobic conditions with agitation at ≥30°C for 4-5 days. There are other variations from this method depending on the desired final flavour as indicated by Thakar and Upadhyay (1992; review). Reduced glutathione intensifies the flavour and stimulates proteolysis, lipolysis and bacterial growth. The ripened slurries can then be used in natural or processed cheeses. Slurries have also been used to screen the suitability of proteinases, lipases and peptidases for accelerating cheese ripening as well as in the production of enzyme-modified cheeses (EMC) to which exogenous enzymes are added. Kristoffersen *et al* (1967) rapidly developed cheese flavour in slurries of fresh curd with various added co-factors or enzymes ripened at elevated temperatures for short periods.

One major drawback of this technology is the difficulty in avoiding the growth of undesirable micro-organisms at the high incubation temperature (≥30°C). This can lead to production of products with unacceptable quality. Roberts *et al* (1995) developed an aseptic cheese curd slurry system for cheese ripening which can overcome the problem of growth of undesirable micro-organisms experienced using slurries. The aseptic slurry system was reported to be suitable only for milk subjected to successive heat treatment or milk treated with hydrogen peroxide to eliminate most of the indigenous milk microflora. Roberts *et al* (1995) however found that milk subjected to severe heat treatments coagulates very slowly and hydrogen peroxide milk produce cheese curd with oxidised flavour hence not suitable for organoleptic evaluations. Natural milk flora which constitutes a portion of
the NSLAB in cheese play some important role in Cheddar cheese flavour development (McSweeney and Fox, 1993). Elimination of such flora in milks to be used for aseptic cheese slurry applications may introduce flavour problems in the cheese produced. Aseptic slurry system application also seems to present difficulty for scaling up due to the need to maintain strict aseptic conditions during operation.

1.4.5 Application of Exogenous Enzymes

Since cheese mature due to the action of indigenous, endogenous and exogenous enzymes rather than their microflora, it appears logical to augment the enzymes known to have definite roles in ripening. Enzyme addition offers a more specific means for enhancement of biochemical reaction rates. That is, there are cheap commercial food grade enzymes that can specifically accelerate either of the two most important reactions, proteolysis or lipolysis, in the maturation of most cheese varieties (Law, 1984). At present there are some drawbacks associated with enzyme addition which include; legal concern in certain countries; limited range of useful enzymes commercially available (Tables 1.1 and 1.2); difficulty in controlling concentration and uniform distribution of added enzymes as well as their activity in cheese; preparation of certain enzymes are expensive (Law, 1984; Fox, 1993a). Law (1995) indicated that the trend in accelerated ripening research is moving away from enzyme addition methods and toward starter culture technology. Nevertheless, the enzyme addition methods have continued to attract more research interests probably because of its simplicity, relative cheapness and the lower risks of product failure as a result of the introduction of combined enzyme systems, Table 1.3 (El Soda, 1993).
Table 1.1 Proteolytic Enzymes Used To Accelerate Cheese Maturation *

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrase</td>
<td><em>Bacillus subtilis</em></td>
<td>NOVO</td>
</tr>
<tr>
<td>Maxatase</td>
<td><em>Bacillus subtilis</em></td>
<td>Gist Brocades</td>
</tr>
<tr>
<td>Maxzyme</td>
<td><em>Bacillus subtilis</em></td>
<td>Gist Brocades</td>
</tr>
<tr>
<td>Rulactine</td>
<td><em>Micrococcus caseolyticus</em></td>
<td>Roussel Uclaf</td>
</tr>
<tr>
<td>Corolase</td>
<td><em>Aspergillus sp.</em></td>
<td>Rohm Tech</td>
</tr>
<tr>
<td>Prozyme</td>
<td><em>Aspergillus sp.</em></td>
<td>Amano</td>
</tr>
<tr>
<td>Acid proteinase</td>
<td><em>Aspergillus oryzae</em></td>
<td></td>
</tr>
</tbody>
</table>

* Adapted from El Soda (1993)

Table 1.2 Lipolytic Enzymes Used to Accelerate Cheese Maturation *

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italase</td>
<td>Animal</td>
<td>Dairyland</td>
</tr>
<tr>
<td>Capalase</td>
<td>Animal</td>
<td>Dairyland</td>
</tr>
<tr>
<td>Kid lipase</td>
<td>Animal</td>
<td>Hansen’s Laboratories</td>
</tr>
<tr>
<td>Lamb lipase</td>
<td>Animal</td>
<td>Hansen’s Laboratories</td>
</tr>
<tr>
<td>Palatase</td>
<td><em>Mucor miehei</em></td>
<td>NOVO</td>
</tr>
<tr>
<td>Piccantase</td>
<td><em>Mucor miehei</em></td>
<td>Gist Brocades</td>
</tr>
</tbody>
</table>

* Adapted from El Soda (1993)

Table 1.3 Enzyme Mixtures Used To Accelerate Cheese Maturation *

<table>
<thead>
<tr>
<th>Name</th>
<th>Enzyme mixture</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naturage</td>
<td>Protease + Peptidase + culture</td>
<td>Miles</td>
</tr>
<tr>
<td>Flavourage</td>
<td>Protease + Lipase</td>
<td>Hansen’s Laboratories</td>
</tr>
<tr>
<td>Accelase</td>
<td>Protease + Peptidases</td>
<td>Imperial Biotechnology</td>
</tr>
<tr>
<td>Flavourzymeb</td>
<td>Protease + Peptidase</td>
<td>NOVO</td>
</tr>
</tbody>
</table>

* Adapted from El Soda (1993)

b From NOVO product sheet
1.4.5.1 Proteinases and Peptidases

The role of proteolysis in flavour development is yet to be understood (Fox et al., 1995) but ironically, research on acceleration of cheese ripening has concentrated on proteolysis of Cheddar. Regardless of the uncertainty over the role of proteolysis in cheese flavour development, there is no doubt that addition of microbial proteinases produces strongly flavoured cheeses within a shortened ripening period (Sood and Kosikowski, 1979). In cheese, proteinases include plasmin, rennet and proteinases of the starter and non-starter bacteria. Peptidases are derived from the cell wall, cell membrane and intracellular areas of the starter and non-starter bacteria.

Proteolysis of casein during cheese ripening is summarised in Figure 1.2. Sood and Kosikowski (1979) with the use of acid and neutral proteinase halved the normal ripening time of Cheddar cheese. Acid proteases however, caused intense bitterness. This was because acid proteinase have greater stability and activity at the pH of Cheddar cheese than the neutral proteinases hence causes more extensive proteolysis. Proteinase-treated cheeses all appear to be crumbly, less elastic and less firm than the controls. This was attributed to the excessive breakdown of β-casein in the cheeses. Such texture defect was not observed in cheeses treated with low level of neutral proteinase. Thus, the selection of proteinase source, type, and amount is critical in order to avoid flavour imbalance and bitter defects (Law and Wigmore, 1982a,b). Law and Wigmore (1982a,b) added neutral proteinase extracted from Bacillus subtilis at a level of 0.0125mg/kg to cheese and found that the flavour intensity was significantly enhanced and the maturation period cut in half. However, addition of either acid (from Aspergillus oryzae) or alkaline (from Bacillus licheniformis) proteinases, or of higher levels of neutral proteinase, produced intensely bitter cheese with little enhancement of flavour. They (Law and Wigmore, 1982a,b) then concluded that increase in excess of 150%, in small peptides and amino acids, of that normally found in Cheddar cheese, irrespective of the enzyme source generally resulted into bitter and meaty flavours.
Vafopoulou et al (1989) used a neutral proteinase, Neutrase, from B. subtilis or an acid proteinase from A. oryzae to accelerate ripening of Feta cheese. Flavour development was accelerated in all treated cheeses and the ripening time was reduced by 50%. Cheeses treated with acid proteinases produced bitterness. Similarly, addition of a lipase/proteinase preparation derived from A. oryzae produced a flavour typical of an aged Cheddar cheese in Colby cheese. On the other hand, the addition of a neutral proteinase produced a texture similar to that of an aged Cheddar cheese without bitter flavour. A lipase/proteinase blend, FlavourAge-FR, has been reported to accelerate the production of good-quality Cheddar cheese (El Soda, 1993). Addition of 48 mg FlavourAge-FR per kg curd at salting was observed to significantly increase water-soluble, ethanol soluble and 5% PTA soluble nitrogen. Flavour and aroma were also improved at low temperature (5°C) but some bitterness was noted. There was reduced flavour score at 10 or 15°C as compared to control and bitterness was noted too. The treated cheeses had soft or brittle body.

Kosikowski and Iwasaki (1975) produced a good quality mature cheese in 3 months at 10°C by adding various fungal proteases and lipases to Cheddar cheese. Higher levels of free amino acids and increased levels of β-casein breakdown was noted in the treated cheeses as compared to the controls. The flavour of processed cheese mix was also enhanced by the inclusion of up to 60% UF retentates which had previously been incubated for 24 h at 45°C with fungal protease or lipase preparation (Sood and Kosikowski, 1979). This allows for the replacement of Cheddar cheese by the enzyme treated UF retentates in the processed cheese formulation.

Law (1980) observed an increase in the levels of soluble-N, peptide-N and amino acid-N over the controls when a preparation of a proteinase/intracellular peptidase(s) from Pseudomonas sp. was added to cheese. When applied at low levels, the intensity and quality of a typical cheese flavour in an 8-week treated cheese was comparable to that of 16-week old controls. The difference in flavour intensity between treated and untreated cheese at week 22 is however very slight. Bitterness and other off-flavours resulted from addition of enzyme(s) at higher levels.
The failure of most exogenous proteinases alone to accelerate cheese ripening which resulted in balanced products switched attention to combinations of proteinases and peptidases, attenuated starter cells or cell-free extracts. Thus Law and Wigmore (1983), in their subsequent work added neutral proteinase in conjunction with a peptidase-rich extract of *L. lactis* spp. *lactis*. The cheese containing the combined preparation showed greater flavour intensity than those without additive or containing only added proteinase or peptidase. Also flavour intensity in cheese with added peptidase were lower than those with added proteinase. Increasing the level of peptidase-rich extract at constant proteinase level enhanced proteolysis but did not increase flavour formation rate. This implied that the transformation of free amino acids to flavour compounds may be rate limiting and that these changes are not catalysed by starter enzymes. This study led to the development of an enzyme system, “Accelase”, for accelerating cheese ripening. Accelase when added to Neutrase-treated Cheddar cheese slurries gives a debittering effect and an intensification of flavour (Cliffe and Law, 1990). Debittering resulted from the sequential production and hydrolysis of bitter hydrophobic peptides giving a predominance of hydrophilic di- and tri-peptides. This means starter peptidases serve to breakdown the large peptides produced by Neutrase and/or chymosin to smaller peptides and free amino acids. Peptidase-rich extracts from *L. helveticus*, *L. bulgaricus* or *L. lactis* were also reported to have accelerated ripening of aseptic, chemically-acidified cheese curds (El Soda et al., 1982). There was an increase in the levels of pH 4.6 soluble N (primary proteolysis) and increased rate of β-casein breakdown. Bitterness was however noted in all of the treated cheeses after two months.

Lin *et al* (1987) compared the effects of Neutrase, calf lipase and a mixture of the two or NaturAge on proteolysis and textural changes in cheese ripened for 16-weeks at 7 or 13 °C. Both 12% TCA- and 5% PTA-soluble N production rates were increased; the latter at a slower rate. NaturAge produced more 5% PTA-soluble N and free amino acids in treated cheeses than the other two enzymes. All enzyme treatments caused softening of the cheeses compared to the controls. Acceleration of ripening and flavour changes were not reported.
A study (Fedrick et al., 1986) indicated that lactose-negative/proteinase-negative (lac'/prt') mutant starter when used in combination with Neutrase reduces bitterness in cheese normally experienced when Neutrase is used alone. This is thought to result from the activity of the intracellular peptidases of the mutant starter. They then suggested that secondary proteolysis or peptidolysis, assessed as 5% PTA soluble-N, seems to be a rate-limiting step in ripening. Similarly, addition of heat-shocked lactobacilli and neutrase to cheese-milk eliminates bitterness noted when the latter is exclusively used and amino N was increased significantly (Ardo and Pettersson, 1988). Increase in amino acid probably results from an increased secondary proteolysis.

Recently the enzyme aminopeptidases of Brevibacterium linens, an important microflora in surface ripened cheeses, was investigated in combination with Neutrase for accelerating cheese ripening (Hayashi et al., 1990a). A significantly higher level of free amino acids was obtained in cheeses treated with this combination than that between Neutrase and peptidase-rich extracts from L. lactis subsp. lactis. Cheese maturation time was also cut by between 50-67%. Although aminopeptidase was very stable in cheese, its distribution was poor and the efficiency of the production method needs to be improved to allow for a better trial. Hayashi et al (1990b) also used seem-purified extracellular serine proteinases from B. linens to accelerate ripening of Cheddar. They observed increase in the levels of TCA-soluble N and flavour development in all treated cheeses kept at 12°C for two months.

Egyptian workers studied the acceleration of Ras cheese ripening by addition of heat-shocked lactobacilli, proteinases/lipases or Neutrase and intracellular enzymes from either L. bulgaricus, Propionibacterium freudenreichii or B. linens. Intracellular enzymes reduced ripening time by up to 25% with that from L. bulgaricus excelling. But addition of Neutrase and intracellular enzymes increased bitterness contrary to the findings of other studies where peptidase-rich extracts reduced bitterness (Fedrick et al., 1986; Law and Wigmore, 1982b; Hayashi et al., 1990). The other findings were similar to those for the earlier studies on accelerated ripening.
Plasmin, is an indigenous milk enzyme which is concentrated in cheese curd and contributes to cheese ripening (Farkye and Fox, 1991). These workers in their investigation found that when cheese milk was supplemented with plasmin, cheese ripening period was shortened (Farkye and Fox, 1992). Plasmin would seem the ideal proteinase for accelerating cheese ripening because it is naturally present in cheese, has narrow specificity, found in close association with the casein hence uniformly distributed in cheese (Fox et al., 1993b). The enzyme however is very expensive at present.
Figure 1.2 Proteolysis of casein during ripening: contributions by rennet, milk proteinase and starter proteinase.
(Adapted from Law and Goodenough, 1995)
rennet  \[\rightarrow\]  CASEIN  \[\rightarrow\]  starter

\[\rightarrow\]  HIGH MOL. WT. PEPTIDES

rennet  \[\rightarrow\]  \[\rightarrow\]  starter (milk)

\[\rightarrow\]  LOW MOL. WT. PEPTIDES

\[\rightarrow\]  \[\rightarrow\]  starter (milk)

\[\rightarrow\]  AMINO ACIDS

\[\rightarrow\]  FLAVOUR AND AROMA COMPOUNDS

(amines, sulphur compounds)
1.4.5.2 Lipases

The acceleration of lipolysis in cheese is quite limited and is usually not rate determining for most varieties with the notable exceptions of the strongly flavoured varieties, Blue and some Italian varieties (Moskowitz, 1980; Jolly and Kosikowski, 1975). The rennet pastes used in the manufacture of certain Italian cheese varieties contain pre-gastric esterase (PGE) activity that is responsible for flavour production. A very similar flavour to that obtained with PGE was obtained by application of an esterase from Mucor miehei in Romano and Fontina cheeses (Moskowitz et al., 1977). Jolly and Kosikowski (1975) accelerated ripening and improved the quality of Blue cheese by using lipase preparation from Aspergillus spp. They observed increased release of typical fatty acid flavour and enhanced production of lactone and ketone precursors. But the lipase preparation had proteinase which led to increased levels of free amino acids.

Egyptian researchers (El Salim et al., 1978) also reported shortened maturation times for Ras and soft pickled cheese respectively when commercial gastric lipases are applied. El Neshawy et al (1982) also accelerated flavour development in Domiati cheese with low levels of pregastric esterase (PGE). Extended ripening however led to the development of rancid off-flavours. Baky et al (1982a,b) while attempting to accelerate the liberation of fatty acids in Ras cheese slurries, reported rapid ripening by adding a preparation containing a Mucor miehei.

There is some concern about the role of lipolysis and fatty acids in hard-cheese flavour. While American researchers reported improved flavour profile in rapidly ripened Cheddar cheese using lipase (Sood and Kosikowski, 1979), English workers failed to develop any such typical flavour while using Mucor miehei lipase (Law and Wigmore, 1982b). The latter researchers reported the production of either no flavour at all or flavour defects characterised as 'soapy' or 'unclean'. Arbige et al (1986) however claimed that lipase secreted by Aspergillus oryzae accelerates the ripening of Cheddar cheese, without off-flavour development.
There is a general belief that the volatile fraction of Cheddar cheese which contains fatty acids contributes a lot to cheese aroma but not to taste. Most of the flavour comes from the non-volatile water-insoluble fraction. Biede and Hammond (1979) indicated that lipolysis has a big role in Swiss cheese flavour mainly as a result of the lipolytic enzymes of the starter cultures.

1.4.5.3 β-Galactosidase (Lactase)

β-galactosidase hydrolyses lactose to glucose and galactose. Addition of β-galactosidase to milk meant for the manufacture of yogurt, buttermilk or Cottage cheese shortened the manufacturing time by 20%. The yield of the Cottage cheese was also increased. This was attributed to the stimulation of lactococci because β-galactosidase shortens the lag period of growth of lactococci. Similarly, a commercial lactase, Maxilact, derived from Kluyveromyces lactis was reported to reduce the manufacturing time, increase proteolysis, improve flavour and accelerate ripening by about 50% of Cheddar cheese (Marschke and Dulley, 1978). Accelerated ripening however was thought to occur due to contaminating protease in the β-galactosidase preparation rather than due to lactose hydrolysis. Infact Maxilact was later (Grieve et al., 1983) shown to contain acid endopeptidase, serine endopeptidase and serine exopeptidase activities and caseinolytic activity.

1.5 Methods of Enzyme Addition

Addition of exogenous enzymes to cheese poses a big challenge to researchers. There are essentially three methods of addition of pure enzymes or enzyme mixtures: addition of free enzymes to milk or to the curd and addition of encapsulated enzymes to cheese milk (Abrahamsen et al., 1989).

1.5.1 Direct addition to cheese milk

Direct addition of enzyme to cheese milk attains a uniform distribution of enzyme throughout the curd. This method also overcomes the low diffusion problem
encountered, due to the large enzyme molecular weight, when added at a later stage (Law, 1984). Direct enzyme addition has some drawbacks (Marschke and Dulley, 1978): both substrates and most of the added enzyme is lost in the whey because proteinases are water soluble making the method uneconomical; the lost enzyme contaminates the whey rendering it unsuitable for other uses and; proteinases can attack the milk proteins in the vat, leading to reduced cheese yields. Losses of enzymes in whey may be prevented by adding them later in the cheese making process, such as the point of salting just before pressing the curd into its final form.

1.5.2 Addition to the Curd

Dried enzyme has been added to the curd before it was pressed and packed (Law and Wigmore, 1982a; Jolly and Kosikowski, 1978; Kosikowski and Iwasaki, 1975). The enzyme powder was 'diluted' in dry salt since the quantity of enzyme used was just a little percentage of the curd to be treated (Sood and Kosikowski, 1979). The method which was meant to overcome the enzyme attack on milk proteins in the vat also resulted in an uneven texture (Law and Wigmore, 1982a). Most modern dairy factories have very large production capacities and produce very large quantity of curd. The large curd sizes in such factories render this technique ineffective (Law, 1980) due to poor migration of the enzyme into the interior of the curd. It also poses health risk through inhalation due to the large quantity of dried enzyme powder to be handled. Other modes of enzyme addition to cheese curd were considered. Lee et al (1978) and Morris (1978) evaluated the potential of injecting enzymes into cheese as a fluid jet. Some loss of the injected fluid occurred, but slow migration of enzymes from the point of injection was probably a greater disadvantage. Low molecular weight products of enzymatic action also diffused readily through the cheese matrix (Lee et al., 1980).

When attempts to add enzyme directly to milk or cheese curd could not produce the desirable results, attention shifted to a method which would confine the enzymes in capsules. The capsules should then be able to release the enzymes or end products into the cheese during ripening. One potential advantage of encapsulation was
envisaged to be the maximum conversion of substrate and reduction in the amount of enzyme and substrate required for the process (Magee and Olson, 1981).

1.5.3 Addition of Microencapsulated Enzymes

Microencapsulation refers to the process of applying a thin film or polymer coat around a small solid particles or gas bubbles or droplets of liquids (Bakan, 1973). The encapsulated material can be released at controlled rates by mechanical fracture, temperature changes, pH manipulation or a combination of such factors. The coated materials, microcapsules, may range from submicron to several millimetres in size and have various shapes, depending on the materials and methods used to prepare them. Most microcapsules have a wall thickness between 10 and 100μm and have a diameter of less than 1 mm (Sparks, 1985).

Microencapsulating technique is employed in the food industry to protect sensitive food components, prevent nutritional loss, preserve flavours and aroma and mask undesirable flavours (Dziezak, 1988). This technique has also been used in cheese technology for accelerating ripening (Kirby and Law, 1987; Law and Kirby, 1987; El Soda, 1986; El Soda et al., 1989; Kirby, 1991; El Soda and Pandian, 1991). Available literature mostly deal with laboratory experiments with few describing pilot-plant experiments. Indications that the method is in commercial use in cheese manufacture is not documented.

Enzyme encapsulation could have the remedy to most of the problems associated with the above mentioned two methods of enzyme addition in cheese. This is because the microcapsules physically separate the enzymes from the substrate in the curd, and only release the enzymes into the curd upon membrane breakdown during ripening (Karel, 1990). Microcapsules also allows for more homogenous enzyme distribution and can be designed for maximum retention in the curd (Law, 1984).

There are many microencapsulation techniques described in the literature. These techniques cover a wide variety of encapsulation materials, which apparently give the
microcapsules particular desired properties. However, only a few of the encapsulation methods have been of interest for dairy applications. Some encapsulation materials and possible methods are reviewed below.

1.5.4 Milk Fat

Milk fat is found in milk or cream as part of an 'oil-in-water' emulsion stabilised by the protein and phospholipid rich milk fat globule membrane, MFGM, (Rajah, 1991). The MFGM can be disrupted either mechanically or chemically to release the milk fat.

Milk fat is composed mainly of triglycerides in nature and its properties depends on the arrangement and number of FFAs within the triglycerides molecules (Banks, 1991). Diglycerides, monoglycerides and FFAs are also present in small quantities mainly as a product of the biosynthesis of milk fat triglycerides and as a result of lipolysis. More than 450 fatty acids have been reported in milk fat (Banks, 1991) although the majority of them are only present in minute quantities.

Milk fat has the most varied chemical characteristics and functional properties among all natural fats. It is a good source of essential fatty acids and has an uniquely pleasing flavour not found in other fats. It is easily digestible due to its higher proportion of short-chain fatty acids (Rizvi and Bhaskar, 1995). However, its high proportion of saturated fatty acids and cholesterol content has limited its direct consumption and or its use as a food processing ingredient because of the potential health risk.

Due to the presence of large variation of its triglycerides, crystallisation and melting of milk fat occurs over a wide temperature range of -40 to 40°C (Rizvi and Bhaskar, 1995). This limits the functional property of milk fat in its natural form. The separation of milk fat into fractions that are considerably different from one another in composition and physical properties and offers attractive possibilities for enhancing the utility of milk fat.
**Milk fat fraction**: Milk fat fractionation allows the separation of fats into fractions with distinct and characteristic composition and melting behaviour. The fat is separated into low melting olein fraction, LMF, and high melting stearin fraction, HMF, (Rajah, 1994).

Several methods have been investigated and used for milk fat fractionation (Rajah, 1994). Milk fat, with or without solvents, were crystallised at different temperatures. The presence of solvents were found to enhance the separation of crystalline and uncrystatallised fat. However, the removal of the solvent posed a considerable problem. Milk fat was also modified by chemical methods such as interesterification and hydrogenation. These methods however caused losses of many desirable characteristics and destruction of natural flavours. The methods produced milk fat fractions of very different physical and functional properties but only minor variations in chemical composition. More recently, the supercritical fluid extraction (SFE) method with CO₂ as a solvent was used by several workers (Shukla et al., 1994). The SFE method was shown to have several desirable characteristics and, among others, resulted in lower cholesterol and saturated fatty acid contents and higher carotene content.

Basically, milk fat fractionation process has two distinct stages (Rajah, 1991): During stage 1 (crystallisation phase), the anhydrous milk fat (AMF) is heated to destroy any crystal nuclei present (60-70°C). The fat crystals are then allowed to develop and grow to ~ 0.2 mm in diameter through controlled cooling. In stage 2 (filtration phase), the higher melting crystals are separated from the lower melting oil when temperature has dropped to below 28°C, melting point of AMF. In contrast to the original milk fat with a melting point of 33 ±1°C, milk fats with melting points ranging from 8-44°C can be obtained (Figure 1.3).
Figure 1.3  Example of a three-step milk fat fractionation
(Adapted from Versteeg, 1991).
BUTTEROIL (100%)
(MELTING Point 33°C)

HARD 1 (25%)  SOFT 1 (75%)
(MELTING Point 43°C)  (MELTING Point 21°C)

HARD 2 (45%)  SOFT 2 (55%)
(MELTING Point 25°C)  (MELTING Point 13°C)

HARD 3 (40%)  SOFT 3 (60%)
(MELTING Point 19°C)  (MELTING Point 8°C)
1.5.4.1 Microencapsulation with Milk Fat

The method of microencapsulation with milk-fat was developed by American researchers (Magee and Olson, 1981 a). They formed microcapsules consisting of milk fat coat containing aqueous protein or glucose. Microencapsulation was done by extruding a water/oil emulsion, consisting of an aqueous solution dispersed in a molten mixture of milk fat and emulsifier, under high pressure through an orifice submerged in a chilled dispersion fluid. This study proved that microencapsulation technique could be adapted and applied to cheese ripening systems. Investigations were then made to develop and optimise encapsulation procedures and to test the system in cheese. Several researchers have since attempted to use milk fat to entrap cheese ripening enzymes (Magee et al., 1981 b; Braun and Olson, 1986a,b). Microencapsulation in milk fat entails enclosing or entrapping the material in the "shell" or matrix of milk fat (Figure 1.4).
Figure 1.4  A schematic drawing of a milk fat-coated microcapsule

(Adapted from El Soda et al., 1989)
buttermilk wall

enzyme

substrate → intermediate product

cofactors and buffers (optimum pH)

flavour compounds

diffusion of products or enzymes
Initial trials of this technique were with flavour generating systems. Magee et al (1981) tested the feasibility of the system by entrapping in milk fat cell-free extracts of *Lactococcus lactis* subsp. *diacetylactis* together with substrates, pyruvate, thiamine pyrophosphate and MgSO₄, and cofactors for diacetyl and acetoin production. The capsules were then incorporated during cheese making. They found the concentrations of diacetyl and acetoin in cheese containing intact capsules increased by eight times that of the cheese prepared without the capsules. This indicated that it was feasible to encapsulate a cheese ripening system that would function in a typical predictable manner.

Braun et al (1982) generated acetic acid from ethanol by encapsulating cell-free extract (CFE) of *Gluconobacter oxydans* with partially purified alcohol dehydrogenase, ethanol (EtOH) and nicotinamide adenine dinucleotide (NAD). They were evaluating the factors that affect the activity of the CFE under conditions which would allow for the gradual synthesis of acetic acid in milk fat microcapsules. In microcapsules with the highest level of encapsulated CFE, acetic acid concentration increased gradually over 40hr to 75 µg/ml of capsules during incubation at 22°C. They also found that conditions, such as pH, can be adjusted to control the formation of acetic acid in milk fat capsules and release in cheese. They however concluded that several factors, such as substrate and NAD concentrations and ratio of NAD to NADH (reduced NAD), need to be considered for the use of this encapsulated system in foods. Amounts of substrate and NAD present control the reaction rate and the final amount of the flavour, acetic acid in this case, produced while NADH is a potential inhibitor of this system. Acetic acid is present in the largest amount of any fatty acid in Cheddar cheese, but excess amounts can cause flavour defects (Dixon et al., 1969). This study had two ramifications; first, that it was feasible to use milk fat microencapsulation technique to generate flavour for enhancing flavour in foods such as cheese and, secondly, that the release of the flavour compounds can also be controlled.

In a subsequent study to characterise a multiple enzyme system from *S. lactis* var *maltigenes*, Braun and Olson (1983) encapsulated the CFE of this microorganism,
leucine and cofactors in milk fat. They then examined how the system's ability to catalyse the formation of flavour compounds, 3-methyl butanal and 3-methyl butanol from leucine was affected by pH, substrate concentration and gradual addition of NADH after incubation at 22°C for 40 h. 3-Methylbutanal and 3-methylbutanol as well as other aldehydes and alcohols derived from amino acids have been identified in cured cheese (Dumont and Adda, 1978). Like in their previous studies, Braun et al (1982), they again demonstrated that the final concentration of the product in the microcapsules depend on the substrate level. Most importantly, they demonstrated that the microcapsules provide good protection to the CFE for at least 12 hr. A fact they established by studying the diffusion of H⁺ across the fat layer of the capsule when the capsules are dispersed in a fluid of various pH values between 4.0 and 8.0. It is important that the capsules provide an isolated environment to ensure not only the protection of products formed like in their studies but also to avoid premature contact between substrate and enzyme, such as during cheese making.

Just like no single compound characterises cheese flavour, the critical compounds and the amounts needed are not known either. It is thus very difficult to select an array of enzymes or CFE that would produce the desired products in appropriate amounts. Microcapsules however helps confine the substrate and the CFE or enzyme in close proximity thus assuring maximum conversion of substrate hence reducing the amount of substrate and enzyme required. Certain flavour producing enzymes require coenzymes which themselves need cofactors for their activities. Cofactors are however expensive (NAD = US $4.00/g, NADH = US $14.00/g) making their application not economically feasible (Braun and Olson, 1986a). Microcapsules was demonstrated by some authors (Campbell and Chang, 1978) that they could be used for regenerating or recycling cofactors relatively cheaply. In a study, Braun and Olson (1986a) successfully enzymatically recycled cofactors and generated acetic acid, 3-methyl butanal and 3-methylbutanol from a mixture of CFE of Gluconobacter oxydans and S. lactis var. malthigenes. The CFE of G. oxydans uses ethanol as substrate to produce acetic acid and to generate NADH from NAD. On the other hand, S. lactis var. malthegenes converts leucine to 3-methyl butanal and 3-methyl butanol, regenerating NAD in that process. These workers (Braun and Olson, 1986b)
then encapsulated in milk fat a mixture of the two CFEs with substrate and NAD. Capsules containing the combined cell-free extracts were added to milk to produce low fat cheese. Upon ripening for 1, 3, and 6 months at 7.2 and 12.8°C, cheeses containing the intact capsules had more 3-methyl butanal and 3-methyl butanol with a stronger malty taste than those with broken capsules or with an incomplete CFE mixture. This again clearly indicated that a wide range of flavours can be produced in foods by multiple enzymatic pathways with the aid of microcapsules. In particular, flavour levels in food products such as cheese can be remarkably raised by such a model system as only a lower level of flavour components can be generated during normal cheese ripening. This can be done by regulating the number of capsules or concentration of substrate.

Also using milk fat, Rippe et al (1983) successfully entrapped partially purified methioninase isolated from Pseudomonas putida, and methionine to produce methanethiol. The reaction mixtures, encapsulated or unencapsulated, were added to milk to produce cheese. Methanethiol production was very high in cheese containing encapsulated enzymes as compared to those with unencapsulated mixtures. The capsules must have provided the close proximity between the substrate and the enzyme necessary for enhancing interaction between them.

Despite the numerous successes with milk fat capsules as in the preceding reviews, the process of forming milk fat-coated capsules has some shortfalls. It poses problems with respect to maintaining enzymatic activities of the carrier mixture because of the relatively high temperature, up to 62°C (Magee et al., 1981), used during the microencapsulation procedure. It was then thought that encapsulating whole cells could overcome this problem. Enzymes in intact cells were expected to have better heat stability than those in CFEs. Based on this argument, Kim and Olson (1985) encapsulated viable Brevibacterium linens with methionine or cysteine in milk fat and successfully produced sulphur compounds. Encapsulation of an intact cell may however pose a problem of difficulty in controlling the reactions and the intact cells may produce both desirable and undesirable enzymes resulting into unwanted products in foods.
The use of milk fat for encapsulation suffers one other big disadvantage; low melting point (33°C). This makes them unstable at cheese cooking temperatures hence unsuitable for Cheddar and related cheese types. When the milk fat capsules are destabilised, the contents are released (Braun and Olson, 1986). The use of higher melting fractions (HMF) of milk fat was suggested (Magee and Olson, 1981) as a remedy to this problem. Braun and Olson (1984) found that milk fat capsules made from HMF exhibited greater stability than those from low-melting fraction of milk fat.

Pannell and Olson (1991a,b) encapsulated in milk fat from HMF pancreatic lipase and/or spores from *Penicillium roqueforti* and very low melting fraction (VLMF) of milk fat. Pancreatic lipase and *P. Roqueforti* spores use VLMF of milk fat as a substrate to produce FFAs and methyl ketones. Methly ketones, important contributors to blue cheese flavour, could enhance flavour in accelerated blue cheese ripening.

In conclusion, encapsulation in milk fat was shown to be feasible and application of the technique in cheese manufacture was successfully tested. The initial encapsulation of a single enzyme was not very successful in producing a balanced cheese flavour because of the complex nature of cheese flavour and the great number of enzymatically catalysed reactions that could potentially affect flavour. Similarly, the encapsulation of a mixture of cell-free extracts with substrates also resulted in an unbalanced flavour. Most of all, the low-melting point of milk fat was its biggest obstacle. The suggestion to use its high melting fraction could be the only remedy if milk fat is to be considered for encapsulating flavour generating systems for cheese applications.

1.5.5 Microencapsulation in Liposomes

Accelerated ripening of cheese has also been achieved by adding liposome encapsulated-enzymes in order to accelerate proteolysis and therefore maturation. Some workers have investigated this method (Skeie, 1994; review). Liposomes are
assemblies of phospholipids and other lipids with a stable biomolecular configuration (El-Soda et al, 1989). Law and King (1985) used Neutrase encapsulated in multilamellar liposomes in Cheddar cheese milk in a laboratory-scale trial. There was no casein hydrolysis after cheddaring as compared to ~20% hydrolysis of β-casein when free Neutrase was used. Overall proteolysis in cheese was very similar whether Neutrase was applied in liposome or in a free form. This showed that the use of liposome was feasible but very low encapsulation efficiency (only 1-2%) and poor retention of liposomes in the curd (17%) discouraged trials for large-scale cheese production.

A better technique, dehydration-rehydration technique, for liposome preparation raised the encapsulation efficiency to 34% and curd retention to 90% (Kirby and Law, 1987). They noted that proteolysis, assessed as TCA-soluble N or as detected by gel electrophoresis, progressed more gradually in the liposome-treated cheeses than in those with free Neutrase. Also unlike in the latter where texture defect occurs within 3 months, the former has normal texture for up to 8 months. Flavour development was accelerated in all treatments.

Spangler and co-workers (1989) prepared liposomes using three different techniques and produced multilamellar vesicles (MLV), small unilamellar vesicles (SUV) or reverse phase evaporation vesicles (REV). They encapsulated Corolase in these liposomes and applied them in milk for Gouda cheese manufacture. REV had the highest proportion of the enzyme encapsulated while SUV had the lowest. Liposome entrapped enzyme had lower proteolysis rate than those of the free enzyme but slightly higher than in the control cheeses. But Alkhalaf et al (1988) had earlier on noted a significant progress in ripening with no textural defects or bitterness when they used REV-entrapped Neutrase during the manufacture of Saint Paulin-type cheese.

Attempts to use temperature-sensitive liposomes containing Corolase in Domiaty cheese milk was not successful. The liposomes were designed for early release of encapsulated enzyme when cheese was held at high temperatures for short periods.
The proteinase were most likely denatured by the high temperatures (50°C for 2 h) employed for encapsulation (El Soda et al., 1989). They then investigated liposomes that may change phase within the pH range of most cheese varieties (5.0-5.5) to release the entrapped enzymes. This sounded more promising but they are yet to publish any progress.

Liposomes thus held lot of good prospects for scientists interested in engineering capsules for enzyme entrapment for accelerating cheese ripening. Liposomes appeared to be easily produced with a wide array of characteristics that could suit different processing conditions found in the hundreds of cheese varieties. This method however suffers from several drawbacks; the ingredients used in these methods are expensive; not generally regarded as safe (GRAS) and edible (Koide and Karel, 1987) and lack suitable method for large scale production. The use of microfluidised liposomes (Lariviere et al., 1991) which does not use organic solvents address these problems but it also suffers from low encapsulation efficiency (12%) as well as big losses (93%) of the encapsulated enzymes during the process. This leaves the field of enzyme entrapment wide open for investigations into other potential encapsulants such as food gums.

Although gums exhibit one the best encapsulating abilities (Dziezak, 1988), there are no substantial documentations on its application to encapsulate enzymes for accelerating cheese ripening. Gums are relatively widely available, cheap, food and biologically compatible. Gum capsules are also easy to prepare. These factors make food gums potential candidates for more investigation into the area of accelerated cheese ripening as encapsulants hence one of the objectives of this research.

1.6 Gums

Gums, also called hydrocolloids, are hydrophilic high molecular-weight polymers. They are polysaccharides with the exception of gelatin. Polysaccharide gums are obtained from plant sources except for xanthan and gellan which are produced by micro-organisms (BeMiller, 1996).
They have individual unique molecular structure which is responsible for their special properties. This forms the basis for their choice and applications in food products.

Gums are often exploited for their texturing capabilities. They dissolve or disperse in water to give a thickening or viscosity-building effect. They are also used for secondary effects which include stabilisation of emulsions, suspension of particulates, control of crystallisation, inhibition of syneresis (the release of water from fabricated foods), encapsulation, and formation of a film (Glicksman, 1979, 1982). A few gums form gels and gelation can be induced thermally or under some special conditions.

1.6.1 Gelation in Gums

The gel-forming gums that are most commonly applied in foods are derived from starch (and derivatives), carrageenan, agar, alginates, gellan, gelatine, pectins, chitosan and cellulose.

What is a gel? Most scientists agree that it is easier to describe a gel than to define it. Classically, gels could be defined as a range of substances that manifest solid-like properties in the presence of a vast excess of solvent (Doublier and Cuvelier, 1996). Gels are quite often soft solids, although harder gels can be made. They can also be deformed (elastic). In terms of chemical constituents, gels usually contain as a major component a substance of comparatively low molecular weight, which in pure form is usually a simple liquid (e.g. water). The elastic character of the gel is due to the presence of a second much higher molecular weight component often present in much smaller relative amount than the ‘solvent’ constituent. This higher molecular weight substance becomes assembled into a full three-dimensional network spanning throughout the entire gel system. This material may be a polymer, or a colloid particle, etc. Thus gels consist of physically immobilised ‘solvent’ similar to a semipermeable membrane through which low molecular weight, e.g. water soluble molecules, can diffuse. For instance, a typical polysaccharide gel contains more than 90% by weight water or aqueous electrolyte (McLoughlin, 1994). The solvent phase (or ‘sol fraction’) of gels may contain other chemical components, including some
polymers or particles which have not become attached to the network. Water can move into or out of the gel depending on the external environment.

Gelation of polysaccharides occur due to cross-linking through polymer-polymer interactions resulting into a network. The networks can be distinguished as a "weak gel" or a "true gel" depending on the macroscopic behaviour (Doublier and Cuvelier, 1996). Generally, a "true gel" should be free-standing and arise from a three dimensional network. For a "weak" gel in contrast, the "solid" nature appears less clearly and tend to flow upon exertion of high enough stress. The transformation from the disordered polymers to a network during gelation is induced by raising or decreasing the temperature (gelatin, carrageenan) or by changing the solvent composition and introduction of specific ions (alginites, low-methoxyl pectins, gellan) (Clark and Ross-Murphy, 1987).

Temperature drop may lead to a change in the polymer conformational state which is often followed by, or directly involves, an association process. This results into gelation if polymer concentrations are sufficiently high. Temperature rise, such as the heat-setting of globular proteins may involve disordering of the polymer and network formation results due to a range of intermolecular interactions including hydrophobic and electrostatic interactions. Clark et al (1990) considered these gels as homogeneous at the molecular level and described them as "association network".

Biopolymer gels can simply be obtained by adding a new component, such as an appropriate salt, to its solution. Changing the pH through addition of acid or alkali may also lead to gelation. Addition of neutral molecules, such as alcohols or urea, and in certain instances, enzymes may induce aggregation. For e.g. casein renneting which involves chemical modification of the original polymer. The presence of other polymers may also induce gelation of another polymer solution. A spectacular example is the gelation of agarose and carrageenans by addition of certain galactomannans.

The added gums may simply drive the conformation change and the association process. This is through altering the strength of interactions within and between
molecules by electrostatic screening of charge or influence on water structure. Added gums may under certain circumstances become directly involved in the association process, for instance calcium ions in certain forms of polysaccharide gelation. Thus the crosslinks between polymer chains in a network originate from physical interactions, which involves such disparate intermolecular forces as Coulumbic, dipole-dipole, electrostatic, Van der Waals, hydrophobic and hydrogen bonding interactions or a combination of these mechanisms. A polymer gel may also be made from an already existing gel by swelling (or de-swelling) it in an appropriate solvent medium.

Gelation properties of polysaccharide is very important especially when the polysaccharide is being considered for use as an entrapment medium for a biological system. It is in the gel structure that the enzymes are immobilised and held. This review focuses on a limited representation of gums that have been used or show potential as good entrapment media.

1.6.2 Carrageenan

Carrageenan is a group of high molecular weight sulphated linear polymer (galactans) extracted from a number of seaweeds (Rhodophyceae, red algae) (Sand and Glicksman, 1973). The carrageenan backbone is based on a repeating disaccharide sequence consisting of D-galactose and 3,6-anhydro-D-galactose units (Therkelsen, 1993) containing 20-40% ester sulphate (CFR, 1992). The repeating units give rise to three major fractions, kappa (κ), iota (ι), and lambda (λ) which differ primarily in the content and distribution of sulphate ester groups. The structure and molecular weight of the fractions determine their functional properties (Marris, 1986).

Carrageenan is soluble in 60-80°C water (Therkelsen, 1994). Iota-carrageenan and kappa-carrageenan can form thermoreversible gels upon cooling of hot aqueous solutions containing various salts (Anonymous, 1991); lambda-carrageenan is nongelling, because of absence of 3,6-anhydro-D-galactose units, but can form viscous solution and acts as a thickener (Rees, 1963). Several investigations have
indicated that helix formation is a prerequisite for carrageenan gelation (Picullel et al., 1989). This notion was however recently challenged by due to the fact that a difference was observed between gelation temperature, \( T_{gel} \), and temperature of onset of helix formation, \( T_o \), for \( \kappa \)-carrageenan. Both \( T_{gel} \) and \( T_o \) are meant to reflect the same molecular event. The carrageenan helix, in spite of disagreement from some scientists, is generally regarded to be a double helix (Picullel, 1996).

Gelation of carrageenan is affected by the presence of ions. It is believed (Piculell, 1996) that carrageenan form ordered helical structure in the presence of cations. Certain cations (\( \text{NH}_4^+ \), \( K^+ \), \( \text{Cs}^+ \), \( \text{Rb}^+ \)) promote both helix formation and gelation, while anions such as \( \Gamma \) and \( \text{SCN}^- \) promote helix formation but impede gel formation. Gelation of sufficiently pure iota-carrageenan is not ion-specific. But normally, iota-carrageenan most strongly gels with \( \text{Ca}^{2+} > K^+ > \text{Na}^+ \) in that order due to the presence of small proportions of \( \kappa \)-carrageenan impurities (Picullel, 1996). With \( \text{Ca}^{2+} \), it forms a clear, soft, resilient and syneresis-free gels (Moirano, 1977).

On the other hand, the lower charged carrageenans, \( \kappa \)-carrageenan and furcellaran, show ion-specificity for their gelation. Kappa-carrageenan is a stronger gelling agent than iota-carrageenan. It gels most strongly with \( K^+ > \text{Ca}^{2+} > \text{Na}^+ \) in the order given. In the presence of \( K^+ \), it produces a strong, rigid, elastic gel which tends toward syneresis, whereas \( \text{Ca}^{2+} \) kappa-carrageenan gives a stiff, brittle gel. The strength of carrageenan gels is also dependent upon the carrageenan concentration (Watase and Nishinari, 1982) and cation concentrations. Gel strength and thermal hysteresis increases with increase in cation concentrations.

Mechanical treatment of \( \kappa \)-carrageenan gel irreversibly destroys it, and a gel is reformed only after melting and re-gelling. Meanwhile iota-carrageenan gels reform upon standing after mechanical treatment (Roesen, 1992).
1.6.2.1 Mechanisms of helical gel formation in carrageenan

The molecular mechanism of gelation of carrageenans have been reviewed by Rees, et al (1982) and Dea (1982). Electron microscopy study suggested that gelation results from the association of polymers into an infinite three-dimensional network. The network structures are branched and associate through a certain length of the polymer, a “junction zone”. Branching results from the incomplete helix formation during chain association or the presence of helix-incompatible ‘kink’ units in the chains. Network formation in carrageenan gels result exclusively from helix association into aggregates and an helical chain may take part in more than one association (Hermansson, 1989).

Mono- and divalent ions influence the coil-helix transition in carrageenan through non-specific electrostatic interactions. Spectroscopic studies have demonstrated that specific cations or anions bind to the helical conformation of κ-carrageenan and furcellaran. These specific ions modify the electrostatic helix-helix repulsion. Specific cations reduce the charge density of the helix considerably and enhances helical aggregation. This is in total contrast to the notion that the specific cations form a sort of ion bridge between the different helices as suggested by other workers (Dea and Rees, 1987). The specific ions conversely increase the charge density and the stability of the helix in solution.

Studies have been conducted on the stability of carrageenan gum powder under acidic or oxidising conditions, high temperature and in the presence of hydrolytic enzymes (Moriano, 1977). However, such studies are lacking for carrageenan gels.

1.6.2.2 Reactivity with Protein

Carrageenans find typical uses in stabiliser blends where it function as a secondary stabiliser, for instance, compensating for whey separation in milk products which may be induced by primary stabilisers (Hansen, 1982). It is also used as the stabiliser of choice for preventing creaming of evaporated milk and infant formulas.
Carrageenans are highly negatively charged macromolecules which can interact with other molecules with opposite charges. Proteins below their isoelectric points carry positively charged groups that complex directly with carrageenan. Cations are required to form an electrostatic bridge between the protein and carrageenan below the isoelectric point. The interaction mechanism critically depends on the stereochemistry of the protein (Tye, 1994). The interaction can lead to precipitation of the protein and carrageenan or to the formation of a complex or gel structure with interesting or useful properties, for instance, in milk based. Complex formation was observed to occur between k-carrageenan and all the main caseins in the presence of calcium and at pH values in the range 6.6-7.0 (Hansen, 1968). Other workers thought whey proteins are co-precipitated along with the casein-carrageenan complex (Manning et al., 1986). A fact exploited in a study to try to increase cheese yield (Kanombirira and Kailasapathy, 1995) by retaining the whey proteins in the cheese curd. Gringrod and Nickerson (1968) further demonstrated that even in the absence of calcium ions, k-casein and k-carrageenan do interact. This was also confirmed in a similar study by Snoeren et al (1975) but who found none of such interactions between k-carrageenan and αs1- or β-casein in a calcium-free system.

The k-casein /k-carrageenan interaction is known to be electrostatic and takes place even though both k-casein and k-carrageenan molecules carry net negative charge at this pH. This has been attributed to the extensive positive charge present on the k-casein from residue 97 to 112, even on the alkaline side of the isoelectric point. This segment on the casein render them capable of electrostatic interaction with the negatively charged sulphate groups of k-carrageenan (Snoeren et al., 1975). On the other hand, αs1- or β-casein lack positively charged segments as their positively and negatively charged residues are more evenly distributed along their polypeptide chains. Κ-Casein in milk is exposed on the surface of the casein micelle and is thus available for interaction with carrageenan (Walstra, 1990).

Carrageenan, however, is known to stabilise calcium-sensitive caseins, αs1- and β-casein, against calcium-induced precipitation (Hansen, 1982). This is important under
conditions where the protective protein, κ-casein, has been destroyed by rennet or homogenisation.

1.6.2.3 Synergistic Interaction with Galactomannans (Locust Bean Gum)

Gums can be used in combination in certain applications since favourable synergistic interactions can occur which result in enhanced rheological properties leading to improvements in product quality. Significant savings in manufacturing costs may also be realised. It has been known for some time that the properties of gel forming polygalactans obtained from red sea weed can be modified by addition of other nongelling polysaccharides, such as the galactomannans. The galactomannans are capable of inducing gelation of otherwise non-gelling concentrations of agars or carrageenans, or give enhanced gel-strength at higher concentrations. They also form mixed gels with xanthan, which is itself non-gelling (Morris, 1990; Dea and Rees, 1987). The most familiar examples of galactomannans are locust bean gum (LBG) and guar gum.

Locust bean (carob) gum is the refined endosperm of the seed of the carob tree, Ceretonia siliqua. It has a linear backbone consisting of D-mannose and D-galactose units in a ratio of ~3.5. Like most galactomannans, it is a neutral polysaccharide. It is only slightly soluble in cold water but totally dissolve when dispersion is heated above 80 °C to give a viscous solution (Dea and Morrison, 1975). Galactomannans are used in a wide range of food products such as dairy, bakery, powdered, jellied, diabetic, beverages, and meat products and seasonings.

Locust bean gum does not form significant gels by itself but will gel in the presence of crosslinkers such as borax (Maier et al., 1993). It interacts synergistically with k-carrageenan and enhances gelation by binding double helices of this polymer in solution into a continuous network. The nature of the synergistic interaction between locust bean gum and k-carrageenan is not clear. It is suggested that the locust bean gum binds to the helical, structure of the k-carrageenan (Tako and Nakamura, 1986). Other workers (Cairns et al., 1988) suggested that there is no specific interaction. But
that the gel network is formed by the carrageenan molecules only which merely entraps the locust bean gum molecules within. The interaction, whatever the mechanism, modifies the texture of the k-carrageenan gel. For instance, pure k-carrageenan forms rigid, brittle, and synerating gels. Addition of locust bean gum results in a gel with greater strength, more resilience, high cohesiveness, and improved water retention.

1.6.3 Agar

Agar is a hydrophilic colloid, like carrageenan, extracted from numerous genera and species of red-purple seaweeds, class Rhodophyta (Selby and Whistler, 1993). Agar is a linear polysaccharide made up of repeating units of disaccharide, galactose and anhydrogalactose, partially esterified with sulphuric acid (Stanley, 1995). The structure of agar consists of two fractions (Araki, 1937): agarose, a neutral polymer that formed a strong gel, and a sulphated nongelling agaropectin. Subsequent studies (Izumi, 1971) revealed that agar is, in fact, a complex mixture of polysaccharides. These polysaccharides consist of charged and uncharged polymers. The latter are unsubstituted while the former are rich in either ester sulphate or pyruvates.

Agar is among the most potent gel-forming agents (Selby and Whistler, 1993), producing measurable gelation at concentration as low as 0.04%. Agar is insoluble in cold water. It dissolves in water upon heating to 85 °C or higher to form a sol. This sol maintains a constant viscosity at 45 °C between pH 4.5 to pH 9.0. Viscosity is also only slightly affected by the presence of ions within the pH range 6.0 - 8.0 at 45 °C. When cooled at 32 - 39 °C, agar sol forms a firm, resilient gel that does not melt below 85 °C (Stanley, 1995). The thermoreversible gel obtained are hard and brittle, and tend to undergo syneresis (Marckhoff, 1987).

The gelling components of agar, which are essentially neutral polymers, require no specific counterions or other additives to induce gelation. However, gelation is known to occur by a similar mechanism to carrageenan gelation (Dea and Rees, 1987). The molecular chains associate into double helices, which then aggregate to form a
network capable of immobilising water (Rees, 1969). This has been confirmed in x-ray studies of agarose fibres (Arnott et al., 1974). The double helix formed during gelation are kept together by hydrogen bonding (Braudo et al., 1991).

The sol-gel transition of agar typically exhibit hysteresis, the melting temperature being higher than the gelling temperatures. Agar gels increase in strength (compressive force required to fracture the gel) on ageing with most of the strength developing within the first 6 hours (Nussinovitch and Peleg, 1990). The gels also synerese on ageing. This phenomenon is due to the contraction of the gel polymer network which decreases the interstitial space available for holding water (Whytock and Finch, 1991).

The ability of agar to bind water and form thermoreversible gels forms the basis of its application in food products. Agar has for a long time been used as the standard culture medium for micro-organisms. It has also been exploited for encapsulating micro-organisms in biochemical reactors (Lebrun and Junter, 1993). In dairy products, it has been used to improve texture and stability of cheeses and fermented products (Selby and Whistler, 1993). Other main areas of food uses include bakery products, confectionery, Japanese desserts and confections, canned meat, fish and poultry products and beverages.

1.6.4 Alginates

Alginates are commercially extracted from the class of brown seaweeds (Phaeophyceae). They were also obtained by bacterial fermentation, but this method is not a commercially viable alternative. The alginate polymer is composed of two building blocks of monomeric units; D-mannuronic acid (M) and L-guluronic acid (G) which are arranged in regions composed solely of one unit or the other referred to as M-blocks and G-blocks and regions where the two units alternate (Haug and Larsen, 1966).
Alginates dissolve in cold water to give a highly viscous solution. Sodium alginate remains disordered in solution, and is used extensively as a thickener. Solubility is however, determined by the pH, ionic strength and gelling ions (Moe et al., 1995) content in the solvent.

Alginates in solution bind multivalent cations. Thus when divalent and polyvalent salts, except Mg$^{2+}$, are introduced into alginate solutions, gels or precipitates are formed instantaneously. Affinity for the multivalent cations depends on their composition; guluronic acid has a characteristic affinity while manurronic acid is almost without selectivity (Moe et al., 1995). The affinity of alginates for cations increases in the order Mg$^{2+}$<Ca$^{2+}$<Sr$^{2+}$<Ba. In other words, affinity is highest for barium and least for magnesium. Reaction with calcium has been more extensively studied due to its usefulness to both food and non-food systems. A wide variety of products can be formulated by carefully controlling the calcium ions. Progressive increase in the concentration of calcium ion added to an alginate solution first leads to an increase in viscosity and ultimately to gel formation. Calcium ions specifically reacts with the G-block regions by binding two chains together tightly to each other, themselves sitting between them like eggs in an egg box.

It is essential to control the calcium ions during the preparation of alginate gels. Because failure to do so will lead to premature gelation and formation of undesirable, broken gels. Gel properties can be controlled by limiting the amount of calcium used; the Ca$^{2+}$ content is commonly expressed as a fraction of the total stoichiometric requirement (i.e. as “percentage conversion”).

In order to avoid the problem of premature gelation of alginates in the presence of calcium ions, alginate gels can be prepared by either of the following three methods; diffusion setting, internal setting and setting by cooling. Diffusion or dialysis setting which is the simplest occurs when gels are formed by allowing calcium ions to diffuse into a solution of alginate. The rate determining step in this method is the time taken for the calcium ions to diffuse through the alginate solution. Since diffusion can be very slow, the suitability of the method is limited to the setting of thin films where
diffusion time is short. This technique has been utilised in the preparation of alginate gels both in the food industry (Cotrell and Kovacs, 1980) and in biotechnology for immobilisation purposes (Skjak- Break and Martinsen, 1991). Diffusion setting method was, for instance, used in this project because of the small size of the gel-beads produced.

Internal setting finds application in circumstances where diffusion setting cannot be used. The technique has also been applied in the food industry (Sime, 1990). Calcium ions are released under control from a calcium salt pre-dispersed in the alginate solution. Factors such as inherent solubility of the calcium salt used, the available amount, particle size and pH determine the rate of calcium release. Others are the inclusion of calcium sequestrants (phosphate, citrate, or EDTA) and the use of slowly dissolving acids (D-glucono-d-lactone, GDL) and short mixing times. The slow hydrolysing lactone releases proton which in turn liberates calcium ions from insoluble salts such as CaCO₃. Slowly dissolving calcium salts such as dicalcium phosphate anhydrous and calcium sulphate dihydrate can be used without acid addition. The rate determining step in this technique is the rate of hydrolysis of GDL and the rate of dissolution of the calcium salt (Sime, 1990).

The last method, setting by cooling, is the least popular of the three methods. It involves the inclusion of the calcium salt in a soluble form in the alginate gel formulation. This method normally gives a weak gel due to the inability to achieve a high degree of conversion even at high temperatures (Sanderson and Ortega, 1994). The gels produced are however thermodynamically more stable than those from the other two methods. This is because, unlike in the other two methods, the calcium required for gelation is present throughout the system in a soluble form.

With calcium, guluronate-rich alginates produce strong brittle gels that experience less syneresis and have excellent heat stability. While the mannuronate-rich alginate produce medium to low, softer and more flexible gels with good freeze-thaw stability but undergo more syneresis.
Alginates can interact with other compounds. They interact with positively charged amino acids residue from denatured proteins and also with highly esterified pectins. The interaction with protein is utilised in the meat industry while that with pectins is utilised in confectionery (Moe et al., 1995). Other properties of alginates important in the food industry include viscosity enhancement, gel formation, stabilisation of aqueous mixtures, dispersions, and emulsions and restructuring food.

1.6.5 Gellan

Gellan gum is a relatively new gelling agent but has been extensively studied. It received food approval in Japan in 1988, and received limited FDA approval for use as a food additive in 1990 (Dziezak, 1990) and a full FDA approval for food use in 1993 (USFDA, 1993). It was approved for food product use in Australia in July 1993 (Anonymous, 1994). It is extracellularly produced by *Pseudomonas elodea* in an aerobic, submerged fermentation.

The gum is a linear, anionic heteropolysaccharide comprising of tetrasaccharide repeating units containing glucose, glucuronic acid, rhamnose and acyl group. In its native form, gellan is acetylated (esterified), but becomes deacetylated (de-esterified) during its extraction. It exists as a co-axial double helix in the solid-state (Chandrasekaran et al., 1988) but undergoes a thermoreversible coil-helix transition in solution (Crescenzi et al., 1987).

Gellan gum requires heating to dissolve. When heated to about 100°C in the presence of ions, it dissolves completely giving a solution of low viscosity. Upon cooling, gelation occurs rapidly resulting into either a thermoreversible or thermo-stable gel depending on the cation concentration (Moorehouse et al., 1981). Gelation occurs only at concentrations greater than 1.2% but in the presence of added cations, both mono- and polyvalent cations, it occurs at unusually low concentrations, typically around 0.2% w/v (Sanderson and Ortega, 1993). Thus, low levels of dissolved calcium prevent chain dissociation or hydration by promoting intermolecular association. However hydration of gellan in such circumstances can be achieved by
inclusion of a calcium sequestrant such as sodium citrate (Bell et al., 1989). Manipulating the relative concentration of the dissolved calcium and sequestrant allows for gellan hydration at room temperature. The divalent ions are much more efficient than monovalent ions at inducing gelation. Gellan gels can be prepared by either diffusion or internal settings. The latter method is more difficult because the use of a sequestrant helps to increase the concentration of sodium in the system. Sodium also promotes the gelation of gellan. Gels are normally prepared by adding the appropriate ion to a hot solution of the gum and cooling. Gellan gels are crystal-clear, and give excellent flavour-release (Morris, 1993).

Robinson et al (1991) suggested that gellan gum exists as a disordered coil at high temperatures. This was in agreement with Grasdalen and Smidsrød (1987) and Crescenzi et al (1987). They, Grasdalen and Smidsrød (1987) and Crescenzi et al (1987), had earlier found out, through physico-chemical studies, that an order-disorder transition occurs in the viscoelastic fluids upon heating and cooling. This was attributed to a helix-coil transition. Cooling of hot gellan solution in the absence of ions results in reversible double helix formation. The helices associate by weak interactions, such as Van der Waals attraction, forming a "weak-gel". However, in the presence of gel-promoting cations, X-ray diffraction studies shows that a proportion of the helices form stronger aggregates.

The formation of helix upon cooling is regarded to promote end-to-end association, via double helix formation, into fibrils. The fibrils then grow thicker into fibre when the end of a chain links to the middle of another chain. Cations which promote gelation induce inter-fibril or intra-fibril aggregation, crystallisation, to give a permanent network (Morris, 1990).

The strength of the gel formed is determined by the cation concentration, as well as the degree of acylation (Kang et al., 1982). At an equivalent ionic strength, divalent cations give rise to stronger gels with higher elastic moduli. But the strongest gellan gum gel is formed under acidic conditions (Grasdalen and Smidsrød, 1987).
Deacylated polysaccharides produce firm brittle gels while acetylated polysaccharides produce soft and elastic gels.

Gellan gum enjoys certain advantages over its rival "older" gums. It is functional at very low use levels; for example, it forms strong gels at concentrations as low as 0.05%. Also by varying its degree of acylation, it can provide a range of textures as opposed to a single characteristic texture (Kang and Petit, 1994).

Gellan has been approved for food use in Japan and the USA with approval being currently sought in the EEC and Canada. It has a potential for application in a variety of food such as confectionery, bakery, meat and dairy products, jams and jellies, and fabricated foods (Sanderson and Clark, 1983). It can also replace agar in a number of Japanese delicacies. Some workers have even demonstrated the potential of gellan for use during microencapsulation (Chilvers and Morris, 1987). A study in this laboratory demonstrated that gellan gum interacts with milk protein and hence helps increase cheese yield (Kanombirira and Kailasapathy, 1995).

1.6.6 Microencapsulation in Food Gums

One of the secondary effects exhibited by gums upon dissolution or dispersion in water is encapsulation (Carroll et al., 1984). Gums seem to be the ideal matrix for entrapment because the crosslinks of their gels provide the essential three-dimensional mesh and network to hold the cell or enzyme in place. This is one property which made gums very popular with numerous workers (Klein and Vorlop, 1985) for immobilising biologically active materials. Gums have been extensively used for the immobilisation of living cells (Willaert and Baron, 1996; review). Similarly, certain enzymes have also been entrapped in the matrices of gums.

Tosa et al (1979) while investigating the applicability of natural polymers for entrapping enzymes successfully immobilised the enzymes aminoacylase, aspartase and fumarase in k-carrageenan matrix. The immobilised enzymes retained high activities and were stable over a long period of use.
Calcium alginate gel has proven to be a reliable, versatile and useful mode of entrapment for both commercial and research purposes (Smidsrod and Skjæk-Braek, 1990). Chang *et al* (1984), co-immobilised oxidase and catalase on calcium alginate beads to convert glucose and oxygen to gluconic acid. Roig *et al* (1995) studied a high-alkaline protease entrapped in an alginate gel. Alginate did not only stabilise the enzyme but to a certain extent also protected it from denaturation in the presence of a detergent. However, increasing the concentration of calcium chloride used for capsule formation decreased the measured enzyme activity. Other workers found that although alginate capsules show high encapsulation efficiency, they are rather leaky (Skeie, 1994), due to the high porosity of the alginate gel (Kierstan *et al*., 1982). This fact could be a great disadvantage in their suitability for entrapping enzymes destined for application in cheese maturation. Premature leakage of enzymes into the cheese curd might result in abnormal cheese.

Prevost and Divies (1988) encapsulated intact cells of *L. bulgaricus* and *S. thermophilus* in alginate beads for fermentation of milk or whey products in a continuous stirred tank bioreactor. They obtained a higher production rate of lactic acid which was 3.3 times that obtained with the free cell mix culture and a short residence time of milk in the reactor.

Greenberg and Mahoney (1981) reported the entrapment of β-galactosidase on agar for the hydrolysis of lactose in milk. De Angelis *et al* (1979) meanwhile immobilised lactase in algin beads. The beads were meant to aid lactose-intolerant people digest lactose in their small intestine when taken in a dose form.

There are very scanty documented reports on the use of gums to encapsulate enzymes for the purpose of accelerating cheese ripening. Schafer (1975) reported in Braun *et al* (1982) investigated the possibility of encapsulating lipases in gelatin capsules for addition to Mozzarella cheese. The encapsulated enzyme was well retained but its release could not be controlled. Law (1984) reported that a worker also attempting to intensify flavour in Mozzarella cheese used formaldehyde-treated gelatin to
encapsulate lipases. But the capsule material failed to melt and release the enzyme in the cheese at the designed temperature (45°C).
1.7 EVALUATION AND SUMMARY OF LITERATURE REVIEW

Cheese maturation process is responsible for the final appearance, sensory and textural properties of cheese (Grippon et al., 1991). However, other factors such as methods of producing the curd and the type of micro-organisms involved also play major roles. Proteolysis during cheese maturation plays an important role in determining the texture of mature cheese (Creamer and Olson, 1982). The extent to which casein and especially $\alpha_\text{s1}$-casein is hydrolysed significantly influence cheese texture (Lawrence et al., 1987). Thus, milk protein or casein, is very important in the development of the final texture of the mature cheese.

During the maturation process, cheese also develop the desirable flavour characteristic to a given variety. The flavour of mature cheese are due to the presence of certain volatile and non-volatile compounds which emanates from the chemical, enzymatic and microbial changes that occur in the lactose, proteins and fats present in the curd (Fox et al., 1995). The contribution of lactose breakdown products to the final flavour of mature cheese is not very clear (Law, 1984). The products of lactose fermentation are important in the flavour of soft-type cheeses which are not matured. Fat breakdown during cheese maturation produces FFAs which are important in surface ripened cheeses such as the Italian varieties. This is because rancid flavour that results from the presence of short-chain FFAs is acceptable in these cheeses (Kilara, 1985). Rancid flavour is not acceptable in Cheddar cheese. Thus, other than the role of fat itself in improving flavour perception, fat breakdown during maturation does not make any significant contribution towards the flavour of Cheddar-type cheeses. Instead the breakdown of fat in such cheeses is avoided through internal ripening and use of weak-lipolytic starter cultures. Proteolysis on the other hand is considered by many researchers as crucial for flavour development in maturing cheeses. Protein hydrolysis produces non-volatile aroma precursors and volatile flavour compounds (Kristoffersen, 1985). One product of protein breakdown that is important for cheese flavour development are amino acids. Amino acids are further catabolised into aroma compounds such as methanethiol and carbonyl compounds characteristic of flavour of Cheddar and other cheese varieties (Manning et al., 1984).
Protein is therefore a major and most important contributor to the flavour and aroma of Cheddar-type cheeses as compared to both fat and lactose. Thus cheese flavour could indicate the extent to which protein hydrolysis has progressed in a maturing cheese and vice-versa. Protein hydrolysis may under certain conditions lead to a bitter flavour. Bitterness normally results from unbalanced proteolysis and influenced by manufacturing conditions, rennet type, microflora and certain added enzymes. It is therefore important to closely control the process of protein breakdown in cheeses in order to achieve a desirable product.

It is however important to note that flavour production in maturing cheese is a very complex biochemical process which involve all the major cheese components such as fat, lactose and protein. The contributions of certain components might be minor but very important for a good quality product. For instance lactic acid is important for texture development while fat is important for flavour perception. Both texture and perception are important either directly or indirectly for the flavour of cheeses.

The protein, and milk fat in curd are degraded during cheese ripening by enzymes (Grippon et al., 1991). The enzymes responsible are either naturally found in milk or are deliberately introduced during the cheese making process. Most of the endogenous milk enzymes are not known to contribute significantly towards the substrate transformation that occur during cheese maturation. This is because most of them get destroyed during milk pasteurisation. Some of them, lipases and proteinases, however survive the HTST pasteurisation temperatures. Lipase activity is not very important in maturation of Cheddar-type cheeses. The milk proteinases, plasmin and cathepsin D, are both known to hydrolyse casein to amino acids and peptides. Their activities may therefore contribute to the process of maturation. However, the contributions of milk endogenous enzymes to cheese ripening process remains minimal when compared to enzymes introduced from outside. This is mainly due to the fact that most of them do not survive milk pasteurisation temperature and those that survive pasteurisation have very low activities. They could however be important in certain traditional cheeses that are produced from unpasteurised milk.
Sources of enzymes that are deliberately added during cheese manufacture include animal stomach, starter cultures, NSLAB and prepared enzymes. Enzyme extracted from the stomach of young animals, rennet, is basically a proteinase called chymosin. Rennet is important for milk coagulation as well as during cheese ripening. Some rennet is destroyed during the initial stages of cheese processing and only a small portion remains in the curd (Fox, 1993a). The residual enzyme activities determine the texture of the mature cheese. Chymosin hydrolyses casein into peptides which are important for flavour development. The LAB from starter cultures release proteolytic enzymes extracellularly during their activities or upon lyses after death. These enzymes degrade peptides into amino acids. LAB also produce lactic acid from lactose fermentation. Lactic acid is responsible for milk coagulation and modifying cheese texture. Lipolytic activity in LAB is very weak but it is a major contributor of lipolysis in internally ripened cheeses. However, rancid flavour normally brought about by lipolysis is not desirable in these type of cheeses. LAB of starter culture is the most important source of enzymes during cheese ripening because of their large number added during cheese manufacture. The occurrence of NSLAB in cheese is more of accidental than deliberate. However, enzymes produced by NSLAB also contribute significantly during cheese ripening. These enzymes metabolise lactate and citrate into flavour compounds, acetate and diacetyl. NSLAB lipase are heat stable and may contribute extensively to proteolysis during maturation, The proteolytic enzymes of NSLAB also degrade the protein in cheese into peptides and amino acids. The contribution of NSLAB enzymes is however relatively lower than that of the LAB of starter cultures but is very important during cheese maturation.

Another possible source of enzyme in maturing cheeses is the addition of prepared enzymes. This kind of enzyme has been added to cheese for the purpose of supplementing the activities of the enzymes usually responsible for cheese ripening. Addition of such enzymes normally lead to the acceleration of the process of cheese ripening. Enzymes that hydrolyse carbohydrates are not known to contribute much towards cheese ripening. Thus most researches have focused on the use of proteases and lipases. Due to the apparent significant role played by proteolysis during cheese maturation, proteolytic enzymes have been preferentially used by researchers in
accelerating ripening. Several of these researchers reported a reduction in cheese ripening period when proteolytic enzymes were used. Initially, researchers used single strain of enzymes such as acid protease which resulted in cheeses of unbalanced flavour and bitterness. Later studies showed that the use of enzyme combinations reduced the problem markedly. Thus subsequent workers have used combinations of proteolytic enzymes. This also led to the development of combined-enzyme commercial brands for use in accelerating cheese ripening. The importance of lipases in the ripening of Cheddar-type cheese ripening is not known. Therefore, most researches on the use of lipases for accelerating ripening has been directed towards the strong flavoured cheeses such as the Italian varieties. Thus with respect to Cheddar and similar cheese varieties, proteolytic enzymes are the most relevant for accelerating maturation. This is due to the fact that the desired flavour and texture of the mature cheese are attributed to the degradation of the protein component.

When adding enzymes to cheese during manufacture, it is important to control their activities to avoid the development of undesired cheese quality. Previous workers have done this by attempting various methods of introducing enzymes into the cheese milk or curd. Addition of enzymes directly into cheese milk achieved a high uniformity in enzyme distribution in the milk. However, the enzymes were immediately exposed to their substrate in the milk and began their activities before curd formation was complete. This led to a poor quality cheese. This method would have best been implemented by use of an inactive form of the enzyme that could be activated during the later stages of curd processing. Attempts by other workers were made to introduce enzymes directly into the curd as dry powder or by injecting liquid enzymes. This was also unsuccessful because of the poor distribution of enzyme within the curd. It resulted into non-uniform maturation in different parts of the cheese. This method was likely to fail because diffusion of enzyme through the curd mass was not going to be fast due to the low liquid or moisture content of the matrix. The method would have probably been successful if several multiple syringes that could reach all parts of the curd were used. This would however be economically not viable. More recently, enzymes were coated in capsules in a process referred to as microencapsulation, prior to application in cheese milk. The technique protected the
substrates in milk from premature enzyme activity. The enzymes were later released gradually in the cheese curd when the capsules broke down. This was a much better technique since the release of enzyme could be delayed until after the formation of the curd.

Generally, milk fat and liposomes have been suggested and tested for encapsulating enzymes. Both milk fat and liposomes enzyme capsules accelerated ripening in cheeses where they were applied. Milk fat capsules in particular were more acceptable because fat is a natural component of milk. It could not however remain intact at curd cooking temperature (≈38°C) which resulted in sudden release of the enzymes. It was suggested that high-melting-point milk fat fraction which does not melt at the curd cooking temperature be used to encapsulate enzymes. However, an appropriate mechanism for the release of the enzyme encapsulated in high-melting-point milk fat fraction capsules should have been devised. One technique would have been to include in the capsules slow-acting lipolytic enzymes that would gradually degrade the capsules to release the other enzymes. Liposomes which are phospholipids were successfully used to encapsulate enzymes. An advantage with liposomes is that they could be produced of different characteristics. The temperature sensitive ones were more popular because curd cooking temperature could act as a trigger to start the enzyme release process. Unfortunately liposome production process involves the use of toxic substances not suitable for food application. Another important encapsulant but which has hardly been used for enzyme encapsulation for cheese application is food gums. Food gums have regularly been used for encapsulating food flavours. Some studies where enzymes have been entrapped in food gums showed that the enzymes retain high activities. Certain gums interact with milk proteins which would make them suitable materials for enzyme encapsulation. The release of enzymes from capsules was however a problem as noted by certain workers who encapsulated enzymes in gelatin and applied them in Mozzarella cheeses. Food gums of plant origin unlike gelatin on the other hand offer a variety of properties. If a proper selection criteria is applied on food gums of plant origin it should be possible to find one with a suitable characteristic for cheese application. The properties of certain gums can also be manipulated to suit certain applications.
and conditions. Thus the release of encapsulated enzymes can be predicted by understanding the prevailing physical and chemical conditions in the cheese.
AIMS AND OBJECTIVES OF THIS STUDY

It is clear that long period of cheese maturation presents an extra cost to cheese manufacturer which could be avoided through speeding up the cheese ripening process. Several methods including addition of enzymes have been investigated, as described earlier, in an attempt to achieve faster cheese ripening. Initial work employed free enzymes but it was established that it was unsuitable due to early proteolysis which resulted in lower yields. Enzymes were later encapsulated in either milk fat or liposomes before applications. Encapsulated enzymes helped to overcome this problem and also reduced ripening period to half in certain applications with less cheese defects. Milk fat however had a lower melting point and also led to earlier proteolysis. Liposomes were expensive and its preparation involved the use of non-food compatible substances.

The aims of the work reported in this thesis were:

1- Examine the feasibility of using food grade gums to encapsulate enzyme
2- Investigate whether these gum capsules can be employed in cheese to accelerate ripening by enhancing enzymatic activity
3- Find out how cheeses ripened by adding the enzyme capsules compare in quality to those ripened normally.

The objectives of the study were:

1- To select appropriate gums for enzyme encapsulation for application in cheese
2- To prepare enzyme capsules using the selected food gums and
3- To produce, with the aid of prepared enzyme capsules, Cheddar-type cheeses within 3-4 months with quality comparable to those ripened for at least 6 months by the normal ripening method.

Before the ultimate aim of using gums as enzyme encapsulant is achieved, it is first necessary to screen some well known food gums for their suitability as potential
encapsulants. Hence, the initial studies outlined in Chapters 2 and 3 were designed to investigate the behaviour of gum gels under simulated cheese manufacturing conditions and their encapsulating potentials. The work that followed (Chapter 4) involved the application of the selected gum capsules in cheese. The trial cheeses were then examined for signs of accelerated cheese ripening to evaluate the effectiveness and feasibility of the overall process.
CHAPTER 2

STUDIES ON SOME PHYSICAL PROPERTIES OF FOOD GUM GELS

2.1 SUMMARY

Gels were prepared from κ-carrageenan, gellan, λ-carrageenan, agar, alginate and κ-carrageenan-locust bean gum. The gels were treated in acidified solutions (0.4, 0.8, 1.2% acidity) and in solutions with varied pH (6.2, 5.5, 4.8). Changes in gel strength of the treated gels were measured after 24 and 48 h using a Texture Profile Analyser (TPA). All treated gels except those of gellan and alginate, showed lower gel strength as compared to the untreated gels or control. Most of the gum gels did not change strength within the solution acidities and pH tested. Only κ-carrageenan gels significantly changed strength when both solution acidities and pH were varied.

The finding from this study indicated that capsules made from κ-carrageenan gum gel are most likely to break down under conditions similar to those encountered during production of Cheddar-type cheese. Thus, κ-carrageenan gum gel exhibited the best characteristics for enzyme encapsulation suitable for application in Cheddar-type cheese.
2.2 INTRODUCTION

A group of materials that are often used for their encapsulating abilities are hydrocolloids or more commonly referred to as food gums. Gums are basically used as texturing ingredients. Gums also possess other capabilities such as encapsulation, stabilisation of emulsions, suspension of particulates, control of crystallisation, inhibition of syneresis and formation of gels (Glicksman, 1982).

Before gums can be used as encapsulants, it is important to study the mechanical properties of their gels under certain conditions of the intended application. In cheese, as in any other foods, the encapsulated enzymes should be released as required. The timing of enzyme release can be controlled by selecting a microcapsule according to its stability properties within a particular food system. A low stable capsule will lead to early release during the food manufacturing process, whereas a more stable one will allow the postponement of its release. This is useful when early release is undesirable and enzyme action is not needed until later in a food manufacture.

For gel-entrapped enzymes to be released, the matrix should get weakened or disrupted to allow the enzyme to escape into the medium or the curd. Thus, gel strength is of great importance for entrapment and release of enzymes. Several mechanisms of release of encapsulated enzymes have been suggested. These include temperature, moisture control, thermal, pH, addition of surfactants, enzyme release and grinding (Karel, 1990). The most relevant mechanisms for the release in cheese could be temperature, pH, acidity and grinding.

In most cases, acidity and pH values of a solution are closely correlated such that if one is known, the other could be precisely predicted. The pH value is a measure of hydrogen ions dissociation in a solution and varies with the solution temperature. Acidity, or titratable acidity, in cheese is expressed in terms of lactic acid produced during the degradation of milk lactose by lactic acid bacteria and other milk constituents such as protein, phosphates and calcium salts (Scott, 1986). Thus, pH value is a measure of the ionic dissociation in a solution and is, therefore, a value
which affects biological or biochemical reactions, as opposed to titratable acidity which relates to the buffer capacity of milk (up to pH 8.3). There is no correlation between pH values and acidity measurements, so that a cheesemaker cannot switch from one form of measurement to another (Scott, 1986). In view of this fact, it is important to study the effect of both pH and acidity on the gel strength of the gums in order to predict the gum capsule behaviour in cheese.

The determination of gel strength as means of assessing the mechanical property of a gel matrix has been used before by several workers (Nussinovitch et al., 1994; Sanderson and Ortega, 1994; Papageorgiou et al., 1994; Sanderson et al., 1988). This technique, called Texture Profile Analysis (TPA), uses an Instron gel tester and can completely characterise a gel (Sanderson et al., 1988). The procedure involves the compression of a free-standing gel disc twice in succession and determining from the force/deformation profile, the textural parameters, modulus, hardness, brittleness, and elasticity. Gel strength is determined as hardness. Hardness is defined as the maximum force that occurs any time during the first compression cycle and usually corresponds to rupture strength.

The objective of this study was to investigate the influence of pH and acidity on the gel strength of the food gums. The pH range selected are similar to those of Cheddar cheese manufacture and the acidity range simulated those encountered during Cheddar cheese manufacture (Fox, 1993a). Lactic acid was added to adjust the acidity of the experimental solutions, because, it is normally produced during cheese manufacture by the starter culture. Lactate is also known to chelate or coordinate Ca$^{2+}$ ions in gel leading to gel disruption (Roy et al., 1987). It was envisaged that this would provide a mechanism for enzyme release in cheese curd. For this same reason, Ca$^{2+}$ has been preferred for gel formation to K$^{+}$ ions which normally gives better gel for carrageenan.
2.3 MATERIALS AND METHODS

2.3.1 Experimental Design

Prepared gels of κ-carrageenan, ι-carrageenan, gellan, agar, alginate and κ-carrageenan-locust bean gums were treated at 3 levels of pH modified and acidified solutions and removed for gel strength testing after 24 and 48 h. Gums were arranged in a Split plot design with four replications. Gum types constituted the main plot. Subplots consisted of the three replicate gum gels corresponding to the three levels of pH and acidity treatments. These were arranged randomly within each main plot.

2.3.2 Materials

Locust bean gum (NP 217 LBG) and κ-Carrageenan (Caragem 107) were supplied by Germantown Company (Sydney, Australia). Agar was supplied by Applied Technical Products Pty Ltd. Gellan gum (Kelcogel) and sodium alginate (Manugel GMB) were obtained from Kelco International. ι-Carrageenan was donated by Swift and Company Ltd. Deionised water (conductivity 0.9 μS), obtained from a Milli-Ro-6 Plus System (hereafter referred to as Millipore water), was used to prepare the gum solutions. Lactic acid, HCl, NaOH and CaCl₂ were all of analytical grade.

2.3.3 Preparation of Gels

2.3.3.1 κ-Carrageenan; ι-Carrageenan and κ-Carrageenan-Locust bean gum (LBG)

Gels were prepared by modified methods of Carroll et al., (1984). Calcium chloride dihydrate (3g) was dissolved in 300mL of Millipore water in a 500 mL beaker. This was then vigorously stirred with a magnetic stirrer and 9.0 g of gum powder added slowly into the vortex and dispersed until a clear dispersion was obtained. This dispersion was then heated while stirring to 80°C and maintained at that temperature for 20 min. to completely dissolve the powder. Stirring was initially done with a glass rod manually when the solution was too thick to be stirred with a magnetic stirrer. The hot solution was dispensed into prepared 25 mL syringes (cut end) and cooled to room temperature before storing them at 6°C for 15 h.
2.3.3.2 Gellan
Gellan gum powder (1.8 g) was dispersed by magnetic stirring in 300 mL of Millipore water, and then heated to 90°C. Calcium chloride (0.06 g or 0.02% w/v) was added to hot gellan solution and the solution filled into 25 mL prepared syringes at 60°C and the gels set by cooling to room temperature. They were then kept at 6°C for 15 h.

2.3.3.3 Sodium Alginate
Sodium alginate gels were made by modifying the method used by Nussinovitch et al (1994). Calcium chloride (0.6g) was dissolved in 300 mL of Millipore water and then heated to boiling point. The boiling solution was vigorously stirred with a magnetic stirrer and 5.4g of sodium alginate powder added slowly and boiled for a further 3 min. The alginate solution was dispensed into prepared 25 mL syringes and cooled to room temperature before transferring to 6°C for 24 h.

2.3.3.4 Agar
Agar gel was prepared according to the modified method of Nussinovitch et al (1994). Agar powder (3.6g) was dispersed in 300 mL of cold Millipore water and heated until a clear solution was attained (80°C for 10 min). The hot solution was dispensed into prepared 25 mL syringes. The solution was then cooled to room temperature and then kept at 6°C for about 15 h.

2.3.4 Treatment of Gels
The set gels were removed from the cooler and left at room temperature for about 2 h. Twenty-one mm length of gels were extruded from the syringes and cut out with a razor blade held against the end of the tube. Three gel segments were placed in duplicate 100 mL beakers each containing 50 mL Millipore water with aciditities 0.4, 0.8 and 1.2% and pH 4.8, 5.5 and 6.2. One set of beakers were kept for 24 h at room temperature while the other set was kept for 48 h at 31°C in a thermostated water bath with occasional stirring. Another set of three gel segments were treated in Millipore water at pH 7.0 (neutral pH) and Millipore water without added lactic acid to constitute the control for the pH modified and acidified solutions respectively.
Solutions of different pH were prepared by adjusting the pH of Millipore water using 0.1N NaOH and 0.1N HCl solutions. These solutions are referred to as pH modified solutions. Acidities of the solutions were adjusted by the addition of lactic acid. To 1100 mL of Millipore water were added approximately 4.8, 9.7, and 14.5 mL of lactic acid to give solutions of 0.4, 0.8 and 1.2% acidity respectively. These solutions are referred to as acidified solutions.

2.3.5 Measurement of Gel Strength

Gel strength of κ- and 1-carrageenan, gellan, agar, alginate and κ-carrageenan-locust bean gum gels were measured using a modified procedure of Sanderson et al (1988). Briefly, the gel segments were removed from the solutions in which they were treated with the aid of a plastic spatula. Gel strength were then measured at room temperature (24-25°C) as the load in g necessary to compress the gel segments to a 25% deformation at a constant rate of 0.3 mm/s with a force of 5 g on the Texture Profile Analyser (TA. XT2, Version 5.15, Stable Microsystems, Surrey, England). The tests were performed at least in triplicate and the gel strength, read as hardness, were calculated from the force-compression curves by a software coupled to the analyser (XT. RA version 3.7).

The choices of the concentrations of the gums and CaCl₂ and the rest of the experimental conditions were based on either previous works or on preliminary studies.

2.3.6 Data Treatment

Data from the four replicates for each gum types and acidity or pH after 24 and 48 h treatment were analysed. In order to examine the changes in gel strength, data analysis was based on gum type and acidity or pH treatment levels and of the gum type and treatment interaction. The significant differences of the means were tested by the Least Significant Difference (LSD) test (Steel and Torrie, 1980).
2.4 RESULTS AND DISCUSSION

In previous studies where gums have been used for encapsulating enzymes or living cells, the emphasis has been on producing a strong and long-term mechanically stable gel capsule (Lacroix et al., 1990; Norton and Lacroix, 1990; Audet et al., 1992). In this study however, the objective was to produce capsules which become unstable during use. Thus, it was not appropriate to adopt the gum concentrations used by previous workers as the aims of those studies were quite different. Gum concentration is one of the key factors that determine the strength, hence stability of the gel capsules produced (Kang et al., 1982). A preliminary study was therefore conducted in order to determine the least concentration of gums that would give a stable capsule. In these studies, the effect of various gum concentrations on the gel strength were investigated. The results obtained indicated that for all the gums tested, gel strength increased with increase in gum concentrations (Figure 2.1). This observation was in agreement with that of Braudo and co-workers (1984). The increase in gel strength with increase in gum concentration was due to a rise in the number of associations among macromolecules. This leads to a progressive cross-linking of the tridimensional network and to a more intense aggregation of the basic structure of the hydrocolloid gels (Rees, 1972). The lowest gum concentrations which gave a firm gel were selected for further trials. The lowest gum concentration should give a gel with the least gel strength, thus enhancing gel breakdown during use.
Figure 2.1 The effect of varying gum and gum concentrations (%w/v) on the
gel strength of: (a) 1-carrageenan, (b) κ-carrageenan, (c) alginate, (d)agar, (e) gellan and (f) κ-carrageenan-locust bean gum
2.4.1 GELS IN ACIDIFIED SOLUTIONS

Generally, treatment of gels in acidified solutions resulted in reduction in gel strength except for gellan and alginate gels at acidities of 0.8 and 1.2% as compared to the control, that is, no lactic acid added both after 24 and 48 h treatments (Tables 2.1 and 2.2). However, the gel strength reduction were not significant (P <0.05) for agar and alginate after 24 h treatment and agar after 48 h treatment. Gellan gel strength increased significantly (P <0.05) after both 24 and 48 h treatments. The greatest reduction in gel strengths after treatment in acidified solutions was observed in κ-Carrageenan, τ-Carrageenan and κ-Carrageenan-locust bean gum as compared to control (Figures 2.2 and 2.3). Varying solution acidities between 0.4 to 1.2% however did not cause a significant (P <0.05) reduction in gel strength of κ-Carrageenan, τ-Carrageenan and κ-Carrageenan-locust bean gum. Upon leaving the gels in the solutions for a further 24 h, κ-carrageenan showed a significant reduction in gel strength but those of τ-carrageenan and κ-carrageenan-locust bean gum (lbg) remained non-significant (Table 2.2).
Table 2.1 The effect of acidity levels on the gel strength of gums after 24 h
<table>
<thead>
<tr>
<th>Acidity (%)</th>
<th>$\kappa$-Car$^2$</th>
<th>Gellan</th>
<th>$\iota$-Car$^3$</th>
<th>Agar</th>
<th>Alginate</th>
<th>$\kappa$-Car-Lbg$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2774.4</td>
<td>1434.1</td>
<td>2569.2</td>
<td>926.2</td>
<td>308.1</td>
<td>982.0</td>
</tr>
<tr>
<td>$\pm$ 221.2a</td>
<td>$\pm$ 162.4a</td>
<td>$\pm$ 75.3a</td>
<td>$\pm$ 163.0a</td>
<td>$\pm$ 41.9a</td>
<td>$\pm$ 97.6a</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>992.3</td>
<td>2368.9</td>
<td>1049.1</td>
<td>896.2</td>
<td>228.8</td>
<td>345.9</td>
</tr>
<tr>
<td>$\pm$ 74.1b</td>
<td>$\pm$ 132.7b</td>
<td>$\pm$ 109.2b</td>
<td>$\pm$ 149.5a</td>
<td>$\pm$ 31.6a</td>
<td>$\pm$ 76.1b</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>958.1</td>
<td>2364.3</td>
<td>1041.2</td>
<td>837.5</td>
<td>478.9</td>
<td>318.9</td>
</tr>
<tr>
<td>$\pm$ 45.4b</td>
<td>$\pm$ 93.2b</td>
<td>$\pm$ 98.5b</td>
<td>$\pm$ 126.8b</td>
<td>$\pm$ 61.4b</td>
<td>$\pm$ 34.2b</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>916.0</td>
<td>2272.2</td>
<td>1021.7</td>
<td>709.0</td>
<td>643.2</td>
<td>305.5</td>
</tr>
<tr>
<td>$\pm$ 48.4b</td>
<td>$\pm$ 85.2b</td>
<td>$\pm$ 130.3b</td>
<td>$\pm$ 85.3b</td>
<td>$\pm$ 48.7c</td>
<td>$\pm$ 43.1b</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Means of four replications; means within column not followed by the same subscript are significantly different at $P < 0.05$.

$^2\kappa$-Carrageenan

$^3\iota$-Carrageenan

$^4\kappa$-Carrageenan-locust bean gum
The observed reduction in gel strength upon treatment in acidified solutions is in confirmation with the idea that presence of lactate, as used in the preparation of the buffer solutions, coordinates calcium ions in the gel leading to its disruption and lowering its strength (Roy et al., 1987). The observed difference between κ- and τ-carrageenan gels could be attributed to the difference in their structures. Both κ-carrageenan and τ-carrageenan are very similar conformationally but have different degrees of O-sulphate group substitution. τ-Carrageenan have more sulphated anhydrogalactose residues in its structure than κ-carrageenan (Therkelsen, 1993). The sulphate groups are thought to be involved in intermolecular interactions through cation bridges in carrageenan gels (Chandrasekaran et al., 1988). This implies τ-carrageenan contains more interchain bonds in its double helices structure than κ-carrageenan, hence more stable. κ-Carrageenan is also known to contain only half the number of interchain hydrogen bonds between galactose residues on the opposite chains of its double helix compared to τ-carrageenan (Chandrasekaran et al., 1988). Thus τ-carrageenan gel is more stable than that of κ-carrageenan as observed in this study.
Figure 2.2 The effect of changes in solution acidity on gel strength after 24 h

Legends: \(\kappa\)-Car - \(\kappa\)-Carrageenan; \(\iota\)-Car - \(\iota\)-Carrageenan;
\(\kappa\)-Car/lg - \(\kappa\)-carrageenan/locust bean gum
Interaction between carrageenan and locust bean gum has been described by Rees (1972) and later confirmed by Ainsworth and Blanshard (1978). They indicated that the smooth regions (i.e. regions carrying no galactose or glucose side-groups) of the locust bean gum molecules bind parallel to double helices of the carrageenan molecules while the branched regions serve as cross-links between molecules. The incorporation of locust bean gum in the carrageenan gel leads to increase in cross-link sites giving the gel more elasticity and more deformation resistance to the system, hence the observed stability in κ-carrageenan-lbg gels in this study.

Gel strength reduction of gellan was not significant after 24 and 48 h (Tables 2.1 and 2.2) when acidity varied between 0.4 and 1.2%. Since gel strength was expected to reduce significantly on addition of more lactic acid, i.e. at 1.2% acidity, due to calcium ions chelation (Roy et al, 1987), the non-significant result could suggest two things. Firstly, the concentration of lactic acid applied was probably not enough to sufficiently coordinate the calcium ions used during gel formation so as to weaken the gel markedly. On the other hand, it could mean that gellan binds calcium ions so strongly, more than alginate or carrageenan, such that a weak chelant such as lactate may not be able to coordinate the calcium ions from the gel matrix. Calcium ions in gellan gel promotes inter- and intra-fibril aggregation which leads to a strong permanent network. It is also important to note that gellan gum, unlike other ion-sensitive gelling polysaccharides, forms gels with a wide variety of ions, including protons (Grasdalen and Smidsrod, 1987). This could as well be the reason why gel strength of gellan gum was not significantly influenced by treatment with acidified solutions.
Table 2.2 The effect of acidity level on the gel strength of food gums after 48 h
<table>
<thead>
<tr>
<th>Acidity (%)</th>
<th>κ-Car</th>
<th>Gellan</th>
<th>1-Car</th>
<th>Agar</th>
<th>Alginate</th>
<th>κ-Car-Lbg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2679.8</td>
<td>1527.2</td>
<td>2347.6</td>
<td>762.9</td>
<td>305.7</td>
<td>919.6</td>
</tr>
<tr>
<td>± 174.0a</td>
<td>±365.9a</td>
<td>±326.9a</td>
<td>±158.3a</td>
<td>±93.9a</td>
<td>±145.3a</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>944.1</td>
<td>2249.8</td>
<td>986.8</td>
<td>754.9</td>
<td>245.7</td>
<td>288.4</td>
</tr>
<tr>
<td>± 46.6b</td>
<td>±273.3b</td>
<td>±167.4b</td>
<td>±107.9a</td>
<td>±29.8b</td>
<td>±43.6b</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>787.5</td>
<td>2424.8</td>
<td>867.4</td>
<td>626.8</td>
<td>524.5</td>
<td>237.7</td>
</tr>
<tr>
<td>± 15.1c</td>
<td>±432.3b</td>
<td>±139.1b</td>
<td>±179.2b</td>
<td>±39.9c</td>
<td>±42.6b</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>384.0</td>
<td>2326.1</td>
<td>877.0</td>
<td>638.8</td>
<td>607.2</td>
<td>225.0</td>
</tr>
<tr>
<td>± 38.6d</td>
<td>±407.5b</td>
<td>±187.8b</td>
<td>±62.2b</td>
<td>±63.3d</td>
<td>±20.0b</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Means of four replications; means within column not followed by the same subscript are significantly different at \( P < 0.05 \).

κ-Car - κ-Carrageenan; 1-Car - 1-Carrageenan; κ-Car/lbg - κ-carrageenan/locust bean gum
When acidity was varied between 0.4 and 1.2%, the reduction in the gel strength of agar gel was not significant after both 24 and 48 h except at 1.2% acidity after 24 h. This could have been due to the nature of the bonding in agar gel. Agar gel formation is not induced by counterions as in carrageenan and gellan gums. Gelation involves hydrogen bonding which makes the crosslinks in agar gels effectively permanent (Lips et al., 1988). Fully set agar gels, irrespective of concentration, do not swell or shrink appreciably when placed in contact with excess solvent at room temperature (Lips et al., 1988). Thus the gels are very stable and are therefore not expected to weaken appreciably in a buffer solution at room temperature.

Alginate gel significantly increased its strength when the solution acidity was increased from 0.4 to 1.2% both after 24 and 48 h treatments (Tables 2.1 and 2.2). This was unexpected as lactate in solution has been reported (Roy et al, 1987) to coordinate calcium ions from alginate gels resulting in gel weakening. So it was expected that an increase in the lactate concentration, higher acidity, would have resulted in a marked drop in the alginate gel strength. Considering this fact, this anomaly was unexpected. The anomaly may however be attributed to the composition of the alginate used. Alginate with high guluronic acid (G) content and long G blocks give alginates with very high calcium reactivity which results in strong gel (Onsoyen, 1994). High calcium reactivity of alginate could diminish the chelating effect of lactic acid. It could also be said that there was probably increase in syneresis in the gel on addition of lactic acid which resulted in an increase in the effective polysaccharide concentration (Nussinovitch et al, 1989) hence higher gel strength.
Figure 2.3 The effect of changes in solution acidity on gel strength after 48 h

Legends: κ-Car - κ-Carrageenan; ι-Car - ι-Carrageenan;
κ-Car/lbg - κ-carrageenan/locust bean gum
There are no reported similar studies on gum gels to which these results could be compared. However, the most significant findings of the experiment on treatment of gels in acidified solutions were firstly, that only κ-carrageenan (Table 2.1 and 2.2) showed a significant reduction in gel strength when treated in solutions with acidity ranging from 0.4 to 1.2%. Gellan, ι-carrageenan, agar and κ-carrageenan-lbg showed no significant reduction in gel strength while alginate exhibited instead a significant rise in gel strength.

2.4.2 GEL TREATMENT IN SOLUTIONS OF DIFFERENT pH

The finding from this experiment indicates that the gel strength of the treated gels were significantly lower (P<0.05) than those of the control (neutral pH) except for gellan and agar where gel strength was not significantly lower after 24h treatment (Tables 2.3 and 2.4).
Table 2.3 The effect of different pH levels on the gel strength of food gums after 24 h
<table>
<thead>
<tr>
<th>pH</th>
<th>κ-Car</th>
<th>Gellan</th>
<th>μ-Car</th>
<th>Agar</th>
<th>Alginate</th>
<th>κ-Car-Lbg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2774.4</td>
<td>1434.1</td>
<td>2569.2</td>
<td>926.2</td>
<td>308.1</td>
<td>982.0</td>
</tr>
<tr>
<td>± 221.2a</td>
<td>± 162.4a</td>
<td>± 75.3a</td>
<td>± 163.0a</td>
<td>± 41.9a</td>
<td>± 97.6a</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>1687.8</td>
<td>1351.8</td>
<td>1309.9</td>
<td>1022.7</td>
<td>28.6</td>
<td>449.4</td>
</tr>
<tr>
<td>± 164.6b</td>
<td>± 182.1a</td>
<td>± 286.8b</td>
<td>± 238.4a</td>
<td>± 5.7b</td>
<td>± 52.3b</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>1703.6</td>
<td>1249.1</td>
<td>1354.2</td>
<td>1112.1</td>
<td>32.2</td>
<td>499.1</td>
</tr>
<tr>
<td>± 298.3b</td>
<td>± 354.5b</td>
<td>± 171.8b</td>
<td>± 246.3a</td>
<td>± 5.2b</td>
<td>± 41.4b</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>1730.1</td>
<td>1348.4</td>
<td>1374.8</td>
<td>1087.1</td>
<td>28.4</td>
<td>491.6</td>
</tr>
<tr>
<td>± 344.0b</td>
<td>± 353.6a</td>
<td>± 337.7b</td>
<td>± 258.0a</td>
<td>± 4.1b</td>
<td>± 78.7b</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Means of four replications; means within column not followed by the same subscript are significantly different at \( P < 0.05 \).

κ-Car - κ-Carrageenan; μ-Car - μ-Carrageenan; κ-Car/Lbg - κ-carrageenan/locust bean gum
Table 2.4 The effect of different pH levels on the gel strength of gums after 48 h
<table>
<thead>
<tr>
<th>pH</th>
<th>κ-Car</th>
<th>Gellan</th>
<th>λ-Car</th>
<th>Agar</th>
<th>Alginate</th>
<th>κ-Car-Lbg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2679.8</td>
<td>1527.2</td>
<td>2347.6</td>
<td>762.9</td>
<td>305.7</td>
<td>919.6</td>
</tr>
<tr>
<td>± 174.0a</td>
<td>±365.9a</td>
<td>± 326.9a</td>
<td>± 158.36a</td>
<td>± 93.9a</td>
<td>± 145.3a</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>2221.8</td>
<td>1292.0</td>
<td>1297.6</td>
<td>1142.5</td>
<td>20.3</td>
<td>462.0</td>
</tr>
<tr>
<td>± 378.3b</td>
<td>± 277.0b</td>
<td>± 214.5b</td>
<td>± 252.6b</td>
<td>± 12.7b</td>
<td>± 89.3b</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>1904.6</td>
<td>1412.1</td>
<td>1298.4</td>
<td>774.8</td>
<td>28.5</td>
<td>459.4</td>
</tr>
<tr>
<td>± 383.1b</td>
<td>± 387.4a</td>
<td>± 233.5b</td>
<td>± 141.7a</td>
<td>± 6.5b</td>
<td>± 76.1b</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>2043.8</td>
<td>1259.0</td>
<td>1285.8</td>
<td>873.9</td>
<td>27.7</td>
<td>524.6</td>
</tr>
<tr>
<td>± 211.4b</td>
<td>± 285.2b</td>
<td>± 420.3b</td>
<td>± 335.3a</td>
<td>± 10.2b</td>
<td>± 70.0b</td>
<td></td>
</tr>
</tbody>
</table>

¹Means of four replications; means within column not followed by same subscript are significantly different at P < 0.05.

κ-Car - κ-Carrageenan; λ-Car - λ-Carrageenan; κ-Car/Lbg - κ-carrageenan/locust bean gum
Gel strength for agar at pH 4.8 also increased significantly after 48h treatment as compared to the control but the increase was not significant for treatments at pHs 5.5 and 6.2. The largest reductions were observed in κ-carrageenan, τ-carrageenan and κ-carrageenan/lbg (Figures 2.4 and 2.5). This suggests that pH treatment has the biggest effect on gel strengths of carrageenan. While the least effects were observed in gellan, agar and alginate.

Varying the solution pH from 6.2 to 4.8 did not cause any significant change in the gel strengths of all the gum gels tested after treatment for 24 h except for gellan at pH 5.5. Similar observations were obtained after 48 h treatment except for gellan at pH 5.5 and agar at pH 4.8. The reduction in gel strength was unexpected because unlike the sols, carrageenan gels are stable at moderately low pH and generally stable at normal food pH at low temperatures (Thomas, 1992). Thus, the difference in gel strength between the gels treated in control solutions and those where pH were varied is unclear. It could however be attributed to the presence of cations, such as Na⁺ ions, in the treatment solutions derived from the NaOH solution used to adjust pHs during preparation of the treatment solutions. Certain monovalent cations including Na⁺ ions can lead to the weakening of gels when present in solutions (Watase and Nishinari, 1984). However, it has also been shown that a reduction in pH increases brittleness by around 10% (Sanderson, 1990). This could lead to an increase in fructurability of the gel which in turn is reflected as a decrease in gel strength.
Figure 2.4 The effect of varying solution pH on gel strength after 24 h

Legends: κ-Car - κ-Carrageenan; τ-Car - τ-Carrageenan;
κ-Car/lbg - κ-carrageenan/locust bean gum
Figure 2.5 The effect of varying solution pH on gel strength after 48 h

Legends: κ-Car - κ-Carrageenan; τ-Car - τ-Carrageenan;
κ-Car/lbg - κ-carrageenan/locust bean gum
Gellan, agar and alginate gels showed no significant change (P<0.05) in strengths after 24 h (Tables 2.3 and 2.4) when the treatment solution pH were 4.8 and 6.2. Gellan and alginate gels also showed no significant difference (P<0.05) in strengths after 48 h. These observations are in agreement with previous views which indicated that gellan, agar and alginate gels are stable over the pH range encountered in many food systems (Gibson, 1992; Sime, 1990; Matushashi, 1990).

It was however noted that the standard deviation of the gel strength determinations for both pH and acidity treatments was very high in certain instances. This was probably due to the sliding of the gel during the compression process on the TPA. When the gels were compressed, most of them exuded water which made the surfaces of the TPA slippery hence the tendency for the gels to slide during compression.
2.5 CONCLUSION

Treatment of gels of κ-carrageenan, gellan, ι-carrageenan, agar, alginate and κ-carrageenan-locust bean gum in either acidified buffer solutions or in buffer solutions with adjusted pH resulted in reduced gel strength except for gellan gum in acidified buffer solutions. Varying the acidity (0.4, 0.8, 1.2%) and pH (6.2, 5.5, 4.8) levels in the buffer solutions however did not produce any significant reduction or change in the gels strengths except for κ-carrageenan. These results indicate that although most of the gum gels tested become weakened by treating in modified solutions, they remain very stable between acidity levels 0.4 and 1.2% and pH 6.2 and 4.8. κ-Carrageenan was the only gum whose gel not only lost strength after treatment but also continued to loose gel strength when acidity and pH levels of the buffer solutions were varied.

κ-Carrageenan showed better potential than ι-carrageenan, gellan, agar, alginate and κ-carrageenan-locust bean gum for breaking down when its gel capsules are treated under conditions similar to those encountered during cheese making. Thus, κ-carrageenan would be the most suitable gum to be employed as enzyme encapsulants for application in cheese.

The determination of gel strengths of the gums after treatments in modified solutions has provided information about the expected stability of the gum gels when used as enzyme encapsulants and applied in cheese milk. In other words, the present study has provided information on which gum has the potential to become unstable when applied in cheese milk and hence release the encapsulated enzyme. In this case, according to the results obtained, the choice would be κ-carrageenan. But other important measures of the feasibility of using gums as entrapment matrices for enzyme is their ability to entrap a lot of enzyme per unit gel weight and even more importantly to maintain the enzyme viability. The next Chapter will discuss the ability of these gums to encapsulate large quantity of enzymes and keep them viable.
CHAPTER 3

ENCAPSULATION OF ENZYME IN FOOD GUM GELS AND
APPLICATION OF THE ENZYME CAPSULES IN CHEESE
MANUFACTURE

3.1 SUMMARY

A commercial proteolytic enzyme, Flavourzyme, was entrapped in κ- and γ-carrageenan, gellan, alginate, agar and κ-carrageenan-locust bean gum gels and made into capsules of diameter 348-524 μm. The specific enzyme activity of the encapsulated enzyme was evaluated. Enzyme encapsulated in κ-carrageenan, gellan and alginate gels showed the highest specific activity, 20.00, 7.69 and 7.14 ΔA_{280}/mgN respectively. Enzyme encapsulated in agar capsules showed the lowest specific activity (1.00 ΔA_{280}/mgN). Enzyme entrapped in these two gums, gellan and κ-carrageenan, showed high activity. Thus, gellan and κ-carrageenan gums had the best characteristics suitable for enzyme encapsulation.

Gellan and κ-carrageenan gums were used to encapsulate enzymes for incorporation into cheese during manufacture. Enzyme capsules were also made from hard milk fat fraction for comparison purposes. Retention of the added enzyme in cheese curd and loss of enzyme in cheese whey were then evaluated. Capsules retention in cheese curd was 91.5, 90.0, and 73.5% for gellan, κ-carrageenan and milk fat respectively. Enzyme losses in cheese whey was 8.86, 5.62 and 17.93% respectively for κ-carrageenan, gellan and milk fat encapsulated enzyme. These observations were comparative to those of liposomes which have been used for encapsulating enzymes for accelerating cheese ripening. Therefore, gellan, κ-carrageenan, just like milk fat which has been successfully used before for encapsulating enzymes, are potentially suitable for encapsulating enzyme for accelerating cheese ripening.
3.2 INTRODUCTION

Effective use of microencapsulated enzyme systems to accelerate cheese ripening requires an efficient method of forming and incorporating stable capsules into cheese and selecting an appropriate array of enzymes (Magee and Olson, 1981a,b). Microcapsules incorporated into cheese must withstand the physical stress and temperatures during manufacture of cheese in order to prevent early proteolysis of milk proteins during milk clotting. They must retain sufficient amounts of enzymes per unit matrix but should also be able to release their enzymatic content during the ripening stage.

Thus for gum-gel encapsulated enzymes to be utilised as a tool to modify enzyme reactions in cheese, the techniques should fulfil certain requirements as indicated by Koide and Karel (1987): a) gel capsule preparation should be mild and simple to prevent enzyme denaturation and to facilitate scale up and; b) capsules should accommodate large amounts of enzymes, that is, encapsulation efficiency (EE: weight % of encapsulated enzyme based on original enzyme weight) must be high, and the ratio of encapsulated enzyme per weight matrix (EPM) should also be high.

Several techniques have been used for entrapping enzymes in gum gels. Gel heating to liquefaction, addition of immobilisant and the solidification by cooling (Kenedy and Cabral, 1983) appeared inappropriate for thermally labile material. Extrusion of a mixture of ionic polysaccharide/immobilisant solution dropwise through a syringe needle into a solution of a divalent cation (Kierstan and Bucke, 1977) was more gentle and simple. However, reduction in bead size is limited by the syringe needle diameter and viscosity of the solution. The syringe-droplet technique was also reported to be unsuitable for industrial scale-up (Poncelet et al, 1992a).

The emulsion techniques where oil-in-aqueous polysaccharide solution is dropped into either cold water (Lacroix et al, 1990), or into CaCl₂ solution (Lim and Sun, 1980) have also been employed for bead formation. Similarly, polysaccharide aqueous solution in oil was added to CaCl₂ solution to form beads (Poncelet et al,
Emulsion technique can produce smaller beads (~200 - 1000μm) and has an unlimited scale-up potential because dispersions can be produced industrially in large equipment. It does not involve toxic reagents or solvents hence suitable for food application. The small sizes of beads produced enhances their uniform distribution within the cheese curd.

Achieving a uniform distribution of encapsulated enzymes in cheese is very important to attain a uniform rate of ripening through out the cheese curd. It is also important that the capsules are retained in the cheese curd in sufficient quantity for the technique to work. A big loss of enzyme capsules into whey may still lead to contamination of the whey and inefficient use of the enzyme. To optimise distribution and entrapment of enzyme-capsules in the cheese curd, earlier researchers (Magee and Olson, 1981; Braun et al, 1982; Braun and Olson, 1986b) have emphasised the addition of enzyme-capsules to cheese milk prior to curd formation. Premature enzyme loss was thereby avoided as most of the capsules were trapped in the cheese curd matrix.

Because of the low specific activity of the enzyme (Kirby et al, 1987), retention of encapsulated enzymes in cheese curd has been determined indirectly. Enzymes were labelled with $^{14}$C and entrapped. Radioactivity was then measured in both the cheese curd and whey as an estimate of the partition of the capsules between these phases (Law and King, 1985; Kirby et al., 1987; Alkhalaf et al., 1988). Unfortunately, the use of radioactive material does not only pose handling hazard, but also requires lots of skills and application of complex equipment. Simple methods for protein quantification such as the modified Bradford method (Bradford, 1976) was used as an alternative to radioactive measurement in this study to try to estimate the retention of enzyme in cheese curd.

The first part of this research was undertaken to evaluate the potential for utilising gum gels as a device for a controlled release of enzyme in the production of cheese. Gum gel capsules/beads prepared by the emulsion technique were examined for their ability to retain enzyme activity. Gum capsule/bead containing the entrapped enzyme
was simply prepared by homogenising a mixture of gum and enzyme solution in vegetable oil.

The second part of this study was conducted with the aim of determining the rate of enzyme retention in the cheese curd to which encapsulated enzyme was applied. Loss of enzyme in cheese whey was also investigated.
3.3 MATERIALS AND METHODS

3.3.1 Materials

The enzyme used for encapsulation was Flavourzyme supplied by Novo-Nordisk A/S (Sydney, Australia). Flavourzyme, developed for protein hydrolysis under neutral or slightly acidic conditions, is obtained by fermenting a selected strain of *Aspergillus oryzae*. It contains both endoprotease and exopeptidase activities (Appendix 1). The activity of Flavourzyme used in this study was 250 LAPU/g where one LAPU (Leucine Aminopeptidase Unit) is the amount of enzyme which hydrolyses 1 µmole of L-leucine-p-nitroanilide per minute.

Soya bean oil and skim milk were purchased locally from a supermarket. Emulsifier used was distilled monoglyceride-Myverol supplied by Swift and Co. Ltd (Sydney, Australia). Casein was obtained from Sigma Chemicals (Sydney, Australia). Protein assay dye reagent concentrate or Bradford reagent containing dye, phosphoric acid, and methanol were obtained from Bio-Rad Laboratories (Sydney, Australia).

Pasteurised (72°C; 15s) non-homogenised milk was obtained from the University of Western Sydney, Hawkesbury dairy factory every morning on the day of cheese making. Frozen concentrated mixed starter culture (*Lactococcus lactis* and *L. cremoris*) for direct to the vat inoculation (DS 5CW3 - 0.1 units) was obtained from Mauri Laboratories (Sydney, Australia). Calf rennet and colorant (Annato) were supplied by Home Cheese Making Supplies (Victoria, Australia). Hard milk fat fraction (melting point 43°C) was donated by the Australia Food Industry Science Centre, AFISC (Melbourne, Australia). Bovine serum albumin (BSA) was purchased from Sigma (Sydney, Australia).
3.4 ENZYME ACTIVITY

3.4.1 Preparation of Enzyme Capsules

3.4.1.1 κ-Carrageenan and ε-Carrageenan

Enzyme capsules were produced by a modified method of Audet and Lacroix (1989). Gum powder (1.5g) was suspended in Millipore water (50 mL). The suspension was heated to 80°C, stirred with a magnetic bar stirrer, and kept at that temperature for 20 min in order to completely dissolve the polymer. The solutions were cooled, ε-Carrageenan to 45°C and κ-Carrageenan to 40°C, and each mixed with 5 mL of a 1% (w/v) Flavourzyme solution by magnetic bar stirrer. The mix was rapidly poured into 150 ml soybean oil containing 0.2% emulsifier in a 250mL beaker kept in a water bath at 40°C. The water-in-oil macroemulsion was formed by stirring with a marine impeller at 2000 rpm. The water-in-oil emulsions were cooled to 25°C by circulating tap water on the beakers to allow the gum droplets to gel. The oil phase was decanted into a beaker and the resulting gel capsules harvested by centrifuging (100xg, 2 min) the emulsion. The gel beads were washed twice with 100mL distilled water and the capsules were separated from the supernatant by sieving on a stainless steel sieve. The gel beads were hardened by soaking in 200 mL of 1% CaCl₂ solution for two hours.

3.4.1.2 Alginate

A modified method of Sheu and Marshall (1993) was employed to prepare alginate gels. Five ml of 1% enzyme solution was mixed with an aqueous solution of alginate (0.9 g) in 50 mL Millipore water. This solution was added drop wise to 150mL of soybean oil in a 250 mL beaker containing 0.2% emulsifier. It was stirred at 2000 rpm by a marine impeller for 1 min. Calcium chloride solution (200 mL, 0.05 M) was added quickly but gently (20 mL/sec) down the beaker side until the water/oil emulsion was broken. The calcium alginate beads/capsules formed were collected by centrifugation (350xg, 10 min). The capsules were then washed twice with 100 mL of distilled water sieved and finally washed with 100mL of 0.05 M CaCl₂ solution.
3.4.1.3 Agar

Agar gel capsules were prepared by the modified method of Nussinovitch et al. (1994). Agar powder (0.6g) was weighed and quantitatively dispersed in 50 mL Millipore water. The dispersion was heated with constant stirring, using a magnetic bar stirrer, to boiling point for 10 min on an electric hot plate. The gel solution was cooled to 40°C and mixed with 5 mL of 1% enzyme solution. The suspension was subsequently dispersed in soy oil (150 mL) containing 0.2% emulsifier while stirring with a marine impeller. Upon formation of droplets, the mixture was cooled by circulating tap water on the beaker until the capsules have solidified. The mixture was transferred to centrifuge tubes and spun down (2 min; 100xg). The separated capsules were washed twice with 100 mL distilled water and sieved.

3.4.1.4 κ-Carrageenan-Locust bean gum (LBG)

κ-Carrageenan-locust bean gum gel capsules were prepared by the modified method of Arnaud and Lacroix (1991). One and a half g of total polymer mixed gel (1.37:0.13 κ-carrageenan:LBG), was dispersed in 50 mL Millipore water. The dispersion was heated to 80°C on an electric hot plate and then cooled to 45°C. A 5 mL 1% enzyme solution was mixed in the warm polymer solution. The mixture was poured into a 250mL beaker filled with 150 mL soy oil plus 0.2% emulsifier at 45°C while stirring at 2000 rpm with a marine impeller to create a strong vortex. The dispersion was cooled to 25°C under tap water until polymer droplets gel while stirring. Formed beads were separated by centrifuging (2 min; 100xg) before being washed twice in 100 mL of distilled water. The beads were separated on a stainless steel sieve and then hardened by soaking in a 1% CaCl₂ solution for 1 h.

3.4.1.5 Gellan

Gellan gum powder (0.3g) was dispersed in 50 mL of deionized water, heated to 90°C with magnetic stirring for 10 min. The solution was then cooled to 45°C and 5 mL of enzyme solution mixed with it and the mixture was poured into a beaker containing 150 mL of soy oil plus 0.2% emulsifier also at 45°C. This polymer-enzyme-oil emulsion is stirred with an impeller stirrer at 2000 rpm for 1 min. The dispersion was then cooled to 25°C with cold water at 20°C to form the gelled polymer beads,
centrifuged (100xg, 2min), washed twice with 100 mL distilled water and sieved. The formed beads were hardened by soaking in 0.07% calcium chloride solution for 2 h.

3.4.2 Determination of gum capsule sizes

The sizes of the beads or capsules produced were determined by the method of Arnaud and Lacroix (1991). One hundred gel beads were first dried on filter paper and the total volume was measured by water displacement in a 25 mL graduated cylinder as follows: Ten mL of water was placed in a 25 mL graduated volumetric flask. One hundred gel beads were added to the water in the flask and the new volume (Vn) read. The average diameter of the beads were calculated from the equation:

\[ d = \frac{3\sqrt[3]{6}Vn - 60}{100\pi} \]

where \( d \) = average diameter of the beads

This equation is based on the assumption that all the gel beads are spherical and the volume (Vs) of a spherical particle is expressed as

\[ Vs = \frac{\pi d^3}{6} \]

Size determination was performed in duplicate for each lot of the manufactured enzyme capsules.

3.4.3 Releasing the Encapsulated Enzyme

In order to determine the encapsulation efficiency and/or enzyme activity of the encapsulated enzyme, the enzymes have to be freed first. This can be done by breaking down the gel matrix that binds them by one or a combination of the following mechanisms; heating, breaking or dissolution (Shahidi and Han, 1993). In
this study, breaking and dissolution techniques were employed to release the encapsulated enzymes because they are relatively mild treatments.

Ten mL of κ- and λ-carrageenan, κ-carrageenan-locust bean gum and gellan gum capsules were dispersed in 50 mL of 0.4% tri-sodium citrate solution and stirred with a magnetic stirrer for 30 min at room temperature (23-24°C) until completely dissolved. Alginate capsules (10 mL) were dispersed and stirred with a magnetic stirrer in 50 mL of 0.1 M phosphate buffer solution (pH 7.3) for 30 min at room temperature until complete capsule dissolution according to the method of Sheu and Marshall (1993). Agar capsules (10 mL) were dissolved by agitating (2000 rpm; 10s) in 50 ml of 0.1M phosphate buffer (pH 7.3). This agitation was repeated about 10 times in order to completely fracture the capsules.

3.4.4 Determination of encapsulated enzyme activity

Activity of the encapsulated enzyme was measured by determining the proteolytic activity of the enzyme on casein as a substrate according to the method of Sarath et al (1989). Casein solution was made by heating a suspension of 2 g casein in 100 mL phosphate buffer (0.1M, pH 7.6) containing 5.25% (w/v) NaCl for 15 min in a boiling water bath until all the casein was dissolved. The solution was cooled and the pH adjusted to 5.2 by adding 0.1 M HCl.

To 20 mL of the casein solution, 5 mL of dissolved enzyme capsules solution was added and mixed thoroughly. The mixture was incubated at room temperature for 20 min. Ten mL of 5% trichloroacetic acid was then added to terminate the reaction. A blank was prepared by combining the trichloroacetic acid and the enzyme capsule solution prior to adding the substrate. The assay mixture and the blank were allowed to stand for 30 min before filtering through Whatman # 42 filter paper. The absorbance of the filtered solution were measured at 280 nm with reference to the blank on a spectrophotometer (Ultrospec-LKB Biochrom 000122, Sydney). Enzyme concentration (total N) in the dissolved enzyme capsules solution was determined by the micro-Kjeldhal method (AOAC, 1990).
One unit of specific enzyme activity was defined as the increase in absorbance at 280 nm (ΔA280) across a 1 cm path length caused by a unit amount of enzyme (expressed as total N) under the conditions of the assay.

3.5 RETENTION OF ENZYME CAPSULES IN CHEESE CURD AND LOSS OF ENZYME IN CHEESE WHEY

3.5.1 Enzyme Capsules Preparation

Three batches of gum capsules having 3 different levels of enzyme concentrations were prepared. A 7.5% solution of Flavourzyme was prepared by dissolving 3.0 g of the enzyme powder in 40 mL of Millipore water at 25°C. The enzyme solution was then used in the production of encapsulated enzymes.

3.5.1.1 Gum Capsules

Gellan and κ-carrageenan gel capsules were prepared as described in Chapter 3 but with changes to the volumes of enzyme solution applied. The different batches of capsules were produced by adding 5.0, 12.7 and 16.2 mL of enzyme solutions to gum solutions in order to obtain enzyme content of approximately 0.34, 1.10 and 2.10 mg per 100, or 83 mL of κ-carrageenan and gellan capsules respectively. These values were a modification of those of Skeie et al., (1995) who successfully applied liposome encapsulated Flavourzyme in cheese to give a final concentration of 0.3 mg enzyme per kg of cheese. The prepared capsules were kept in CaCl₂ solution for 1 hr, washed in 200 mL cold distilled water before application to cheese.

3.5.1.2 Hard Milk Fat Fraction Capsules

Hard milk fat fraction microcapsules were produced according to a modified method described by Magee and Olson (1981). Briefly, 150 g of hard milk fat fraction was melted in a steam water bath and heated further to 62°C. Emulsifier (0.25%) was
added and stirred with a magnetic stirrer. The hard milk fat fraction and emulsifier blend was cooled to 38°C under tap water. The prepared enzyme solution was added while stirring with a magnetic stirrer at one of the three levels, 5.0, 12.7 or 16.2 mL, depending on the trial cheese being made. The hard milk fat fraction-enzyme mix was then added while stirring (1200 rpm) to 250 mL clean distilled water (10°C) in a beaker to form the fat capsules. The capsules were left in cold water to stabilise for 30 min. The capsules were separated by centrifuging (100xg; 3 min) washed twice in 250 mL of cold distilled water (10°C), kept for 1 hr in cold water, sieved on a stainless steel strainer (120 μm pore diameter) before incorporating into cheese milk.

The enzyme capsules of different levels were prepared by varying the quantity of enzyme solutions added to gum solutions as indicated in Table 3.1(a) below.
Table 3.1(a). The volume of enzyme solution added to 200 mL of
gum solution and molten milk fat during preparation of the
different types of enzyme capsules
<table>
<thead>
<tr>
<th>Level</th>
<th>Encapsulant Type</th>
<th>Volume of enzyme (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>κ-Carrageenan (Ki)</td>
<td>5.0</td>
</tr>
<tr>
<td>Medium</td>
<td>κ-Carrageenan (Km)</td>
<td>12.7</td>
</tr>
<tr>
<td>High</td>
<td>κ-Carrageenan (Kh)</td>
<td>16.2</td>
</tr>
<tr>
<td>Low</td>
<td>Gellan (Gl)</td>
<td>5.0</td>
</tr>
<tr>
<td>Medium</td>
<td>Gellan (Gm)</td>
<td>12.7</td>
</tr>
<tr>
<td>High</td>
<td>Gellan (Gh)</td>
<td>16.2</td>
</tr>
<tr>
<td>Low</td>
<td>Milk fat (Mi)</td>
<td>5.0</td>
</tr>
<tr>
<td>Medium</td>
<td>Milk fat (Mm)</td>
<td>12.7</td>
</tr>
<tr>
<td>High</td>
<td>Milk fat (Mh)</td>
<td>16.2</td>
</tr>
</tbody>
</table>
3.6 Cheese Making

The control Cheddar cheese (no enzyme added) was manufactured according to the method described by Australian Society of Dairy Technology (1977) as indicated in Figure 3.1 using pasteurised milk (72°C, 15s) with 2% starter culture and 0.25% (v/v) rennet. The pasteurised milk was standardised to a casein:fat ratio of 0.71 using skim milk. Annato and CaCl₂ solutions were added at a rate of 0.25% (w/v) each. Cheese manufacture was done in a 10 L water-jacketed vat fitted with a variable speed agitator blade (Armfield FT20-A, Ringwood, England). After milling the curd, salt (NaCl) was applied at a rate of 2.5% (w/w) to the curd. The curd was pressed with an 8 kg weight stone overnight (≈16 h), packaged in cryovac film and kept in a cheese room (8°C) to ripen. Three batches of the control cheeses were produced in similar ways.

For the experimental Cheddar cheeses (enzyme capsules added), a similar procedure was followed as for the control cheese except for the introduction of the enzyme capsules. The enzyme capsules made with gellan, κ-carrageenan gums and milk fat were introduced into the cheese milk at 32°C just before the addition of rennet according to the protocol in Figure 3.1. Every gum and the milk fat had capsules prepared with three levels of enzyme concentrations to give three treatment levels for each gum and the milk fat (Table 3.1b). Stirring was continued after enzyme capsules addition up to the point of rennet addition. After that, the same procedure as used in control cheese manufacture was exactly followed.
Figure 3.1 Flow process chart for cheese manufacture
Pasteurized Milk (10 L)

Annato (0.25%)

Starter (2.0%)

Enzyme capsules

Rennet (0.25%)

Agitation (31°C)

Agitation (2 min)

Agitation (3 min)

Agitation (2 min)

Setting (31°C; 45 min)

Cutting

Cooking (38°C; 40 min)

Whey Drainage (1.2% acidity)

Cheddaring (85 min)

Salt (2.5%)

Salting, Milling, Pressing (0.55% acidity)

Maturation (8-10°C)
Table 3.1 (b). The quantity of enzyme added to cheese milk at each level of capsule preparation
<table>
<thead>
<tr>
<th>Level</th>
<th>Encapsulant Type</th>
<th>Volume of capsules added to cheese milk (mL)</th>
<th>Equivalent weight of enzyme added to cheese milk (mg)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>(\kappa)-Carrageenan (Kl)</td>
<td>100.0</td>
<td>0.342</td>
</tr>
<tr>
<td>Medium</td>
<td>(\kappa)-Carrageenan (Km)</td>
<td>100.0</td>
<td>1.036</td>
</tr>
<tr>
<td>High</td>
<td>(\kappa)-Carrageenan (Kh)</td>
<td>100.0</td>
<td>2.035</td>
</tr>
<tr>
<td>Low</td>
<td>Gellan (Gl)</td>
<td>83.0</td>
<td>0.367</td>
</tr>
<tr>
<td>Medium</td>
<td>Gellan (Gm)</td>
<td>83.0</td>
<td>1.025</td>
</tr>
<tr>
<td>High</td>
<td>Gellan (Gh)</td>
<td>83.0</td>
<td>2.127</td>
</tr>
<tr>
<td>Low</td>
<td>Milk fat (Ml)</td>
<td>48.0</td>
<td>0.359</td>
</tr>
<tr>
<td>Medium</td>
<td>Milk fat (Mm)</td>
<td>48.0</td>
<td>1.145</td>
</tr>
<tr>
<td>High</td>
<td>Milk fat (Mh)</td>
<td>48.0</td>
<td>2.206</td>
</tr>
</tbody>
</table>

\(^1\)Means of two determinations by the micro-Kjeldhal method.
3.6.1 Retention of Enzyme Capsules in Cheese Curd

Capsule retention in cheese curd was measured indirectly by determining the quantity of capsules lost in the cheese whey. The entire amount of cheese whey was collected during the manufacturing procedure. The whey was then strained in a 120μm stainless steel sieve. The capsules collected on the sieve was collected and its volume measured in a 50 ml measuring cylinder. Retention of enzyme capsules was then expressed as a percentage of the total volume of capsules applied in the cheese milk.

\[
\text{Retention} = 1 - \frac{\text{Volume of capsules collected from whey (mL)}}{\text{Total volume of capsules added to cheese milk (mL)}} \times 100\%
\]

This method was considered appropriate due to the fact that the enzyme capsules added to the cheese milk was unlikely to rupture during cheese processing. Capsules rupture would not occur because the highest curd cooking temperature of 38°C was neither high enough to melt the hard milk fat fraction capsules nor the gum capsules. The enzyme capsules may only rupture during the pressing of the cheese curd. Another precaution taken to avoid rupturing the enzyme capsules during the cheese making process was by stirring the curd very slowly and gently in the cheese vat during cooking.

3.6.2 Enzyme Losses in the Cheese Whey

Enzyme losses from gum capsules during cheese manufacture were determined as the increase observed in the protein content in the experimental cheese whey. The increase was calculated as the difference between the protein content in the control cheese whey and the experimental cheese whey and expressed as a percentage of the protein content of the capsules applied to cheese milk.
Enzyme Loss = \frac{WT - WC}{P} \times 100\%

where; WC is protein content of control cheese whey
WT is protein content of trial cheese whey, and
P is total protein content of capsules applied to experimental
cheese milk.

Total protein content of capsules and protein content of whey were determined by a modified Bradford method (Bradford, 1976) supplied by Bio-Rad Laboratories, Australia. Gellan and κ-carrageenan gum capsules were solubilised as indicated in chapter 3, Section 3.2.4. Milk fat capsules were melted by heating in a beaker of boiling water. Five dilutions of bovine serum albumin (BSA) protein were prepared to give protein concentrations of 0.02, 0.04, 0.06, 0.08 and 0.09mg/ml for the preparation of a standard curve. Protein content determinations were then carried out as indicated in chapter 3, Section 3.2.5 in a water bath at 45 °C to prevent the milk fat solidifying.

3.7 Statistical Analysis

Analysis of variance were carried out using a computer statistical package, Microstat (Ecosoft, Inc., IN, USA) and the significant differences of means confirmed by Duncan’s Multiple Range Test (Steel and Torrie, 1980).
3.8 RESULTS AND DISCUSSION

3.8.1 Enzyme Activity

Results for the determination of enzyme activity, expressed as the specific enzyme activity, of the released enzyme are indicated in Table 3.2. Specific enzyme activity is the catalytic activity of the enzyme that is related to its protein content (Gerhartz, 1990). The enzyme activity for enzymes released from κ-carrageenan, capsules was significantly different (P<0.05; Table 3.2) from those released from other gum capsules. However, there were no significant differences between the specific enzyme activities of enzyme released from gellan and alginate capsules; from τ-carrageenan and κ-carrageenan/βlg capsules and, from κ-carrageenan/βlg and agar capsules. κ-Carrageenan entrapped enzyme showed the highest specific enzyme activity while agar encapsulated enzyme exhibited the lowest specific enzyme activity.
Table 3.2 The effect of encapsulation with gum gels on enzyme activity
<table>
<thead>
<tr>
<th>Gum</th>
<th>Temperature (°C)(^1)</th>
<th>Capsule size (μm)(^2)</th>
<th>Specific enzyme activity (ΔA₂₆₀/mg N)(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ι-Carrageenan</td>
<td>45</td>
<td>479.2</td>
<td>2.04c</td>
</tr>
<tr>
<td>κ-Carrageenan</td>
<td>32</td>
<td>430.8</td>
<td>20.00a</td>
</tr>
<tr>
<td>κ-Carrageenan/Sp</td>
<td>45</td>
<td>445.5</td>
<td>1.35cd</td>
</tr>
<tr>
<td>Gellan</td>
<td>47</td>
<td>371.6</td>
<td>7.69b</td>
</tr>
<tr>
<td>Agar</td>
<td>35</td>
<td>348.1</td>
<td>1.00d</td>
</tr>
<tr>
<td>Alginate</td>
<td>25</td>
<td>524.7</td>
<td>7.14b</td>
</tr>
</tbody>
</table>

\(^1\) Temperature of gum solution when enzyme was introduced

\(^2\) Means of duplicate determinations.

\(^3\) Means of duplicate determinations. Values followed by the same letter are not significantly different at P < 0.05.
Although temperature is one of the factors that govern enzyme catalytic activity (Gerhartz, 1990), the variation in the specific activity of enzyme encapsulated in different gum capsules could not be attributed to temperature. Optimum temperature increases the reaction rate thermodynamic but above optimum, it causes thermal degradation of the enzyme. But as indicated in Table 3.2, enzyme introduced in agar solution at 35°C showed lower specific activity than enzyme introduced in gellan gum solution at 47°C. This could have been the case because the enzyme used, Flavourzyme, was indicated by its manufacturer, Novo-Nordisk, as having an optimum temperature of activity of 50°C hence was not affected under this experimental conditions.

The most probable explanation for the observed differences in specific enzyme activity could be related to the differences in the gums used. Some of the gums used in this study, such as κ-carrageenan, gellan, ι-carrageenan, and alginate are polyanionic polymers, i.e. they contain large numbers of charged groups. These groups have substantial effects on ionic balance because they tend to concentrate protons in solution which alters the solution's pH (Cornish-Bowden, 1985). The change in pH affects the activity of the entrapped enzyme. The differences observed in specific enzyme activity for the different gum capsules in this study, must have been a result of differences in the number of charged groups that they carry. κ-Carrageenan carry relatively lower number of charges (Watase and Nishinari, 1982) as compared to the other gums. Thus, its presence could have affected the solution pH least hence the high enzyme activity. The high specific enzyme activity of κ-carrageenan encapsulated enzymes also reflected the ability of κ-carrageenan gels to break down easily when solution pH and acidity are varied (Chapter 2) as compared to the other gums.

Another important factor is the fact that enzymes encapsulated in charged matrices, as in this study, are subject to significant electrostatic effect when changes in ionic strength of solution occurs (Kilara and Shahani, 1985). Change in solution ionic strength causes change in enzyme activity and usually lead to over estimation of activity at high ionic strength (Kilara and Shahani, 1985). Thus the introduction of
calcium ions for hardening capsules of certain gums in this study must have affected activity of the entrapped enzyme. Differences in activity for enzyme entrapped in the different gums was probably due to the different strengths of calcium chloride solution used. Because agar is a neutral polymer and does not require cations to gel, it can be inferred that both polyionic polymers and high ionic strength positively influenced enzyme activity.

Again, there are no reported similar studies with which to compare the findings of this work. Nevertheless, this study has shown that κ-carrageenan, gellan and alginate encapsulated enzymes have shown a significantly higher specific enzyme activities than those of agar, τ-carrageenan and κ-carrageenan-locust bean gum.

3.8.2 Retention of Capsules in Cheese Curd

One main factor that determines the amount of proteolytic activity available for casein degradation is the level of capsule retention in the cheese curd. The rate of capsule entrapment in the cheese curd is also another factor which significantly influences the cost of using enzyme capsule in cheese ripening. High level of capsule retention would therefore not only guarantee increased proteolytic activity but also makes the whole process of enzyme encapsulation economical.

Table 3.3 shows the results of the retention of gellan, κ-carrageenan and milk fat capsules in cheese curd. All the three encapsulants showed very high entrapment rates. The retention rate for the two gum capsules, κ-carrageenan (90.0%) and gellan (91.5%) were not significantly different but were significantly higher than that of milk fat (73.5%) capsules. The retention rates of the two gums are comparable to that of dehydrated-rehydrated (DRV) liposomes of 90%, the highest reported for liposomes (Kirby et al., 1987). The retention rate values for both gums and milk fat in this study were also higher than those observed by Alkhalaf et al., (1988) for free Neutrerase in cheeses which was only 20%.
Table 3.3. Retention of Enzyme Capsules in Cheese Curd.
<table>
<thead>
<tr>
<th>Capsule Type</th>
<th>Capsule size (μm)</th>
<th>Capsule Retention (%)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>κ-Carrageenan</td>
<td>473.6</td>
<td>90.0a</td>
<td>2.11</td>
</tr>
<tr>
<td>Gellan</td>
<td>389.1</td>
<td>91.2a</td>
<td>2.36</td>
</tr>
<tr>
<td>Milk Fat</td>
<td>658.2</td>
<td>73.5b</td>
<td>7.46</td>
</tr>
</tbody>
</table>

1 Means of duplicate determinations

2 Means of the three levels of capsules applications; each determined in duplicate.

Values with different subscripts differ significantly at P < 0.05.
Unlike for liposomes where smaller sized liposomes were poorly retained in the cheese curd in comparison to larger sized liposomes (Kirby et al., 1987), gum capsules in this study had smaller sizes than milk fat capsules (Table 3.3) but exhibited higher retention. Thus, capsule size did not affect capsule retention rate in cheese curd in the present study. The difference in the retention rate between the two gum capsules and milk fat capsules observed could be due to the difference in the weight or density of the capsules. The gum capsules being higher in density (not reported) would tend to remain in the cheese milk during cheese making and get trapped in the curd matrix when milk sets. A proportion of the less denser milk fat capsules tended to float (personal observation) and therefore had a lower chance of being trapped in the curd matrix.

Both gellan and κ-carrageenan gums are known to interact with proteins (Sanderson and Clark, 1983) and this could be another reason for the higher retention observed for their capsules since they could have interacted with casein in milk.

3.8.3 Loss of Enzyme into Cheese Whey

Loss of enzyme in cheese whey does not only lead to the contamination of whey but may also raise the cost of the operation. When enzyme leaks prematurely from the capsules during cheese manufacture, it could mean the encapsulation process was inefficient. A well engineered capsule should release its enzyme content only when desired (Karel, 1990) and thus minimise enzyme losses.

Enzyme losses from gellan, κ-carrageenan and milk fat capsules in this study are presented in Table 3.4. In general, the biggest loss was experienced from milk fat capsules followed by κ-carrageenan and then gellan. The losses in κ-carrageenan was significantly higher than those of gellan at all levels of enzyme concentrations in the capsules. The losses of enzymes for all types of capsules were well lower than those reported (80%) for a free protease, Neutrase, applied to cheese (Alkhalaf et al., 1988). This points to the fact that encapsulation in gellan, κ-carrageenan or milk fat remarkably reduces losses of enzymes into cheese whey. The significant difference in
losses observed between gellan and κ-carrageenan made capsules (Table 3.4) is probably due to the extent to which the two types of capsules get weakened in the presence of lactic acid. Lactic acid coordinates $\text{Ca}^{2+}$ in the gel (Roy et al., 1987) and disrupts the gel structure which leads to enzyme leakage from the capsules. It can be argued from this finding that $\text{Ca}^{2+}$ in κ-carrageenan gel is easily co-ordinated by lactate than those in gellan gel. Gellan gum reacts with a large variety of other ions (Grasdalen and Smidsrod, 1987) to form gel and this can possibly occur in the complex milk system. Thus even if the $\text{Ca}^{2+}$ ions used during capsule preparation is co-ordinated by lactate, the capsule still remains stable because of its interaction with other ions. Hence the observed low level of enzyme leakage from the capsules in this study.
Table 3.4. Enzyme Loss in Cheese Whey
<table>
<thead>
<tr>
<th>Capsule Type</th>
<th>Enzyme Loss in Whey (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>k-Carrageenan</td>
<td>8.66a</td>
<td>2.13</td>
</tr>
<tr>
<td>Gellan</td>
<td>5.62b</td>
<td>2.17</td>
</tr>
<tr>
<td>Milk Fat</td>
<td>17.93c</td>
<td>1.96</td>
</tr>
</tbody>
</table>

<sup>1</sup> Results are given as means ± standard error of the three levels of enzyme applications; each level determined in duplicate. Values with different subscript differ significantly at P < 0.05.
The significantly higher enzyme losses in milk fat capsules treated cheese could be attributed to either the melting of the milk fat capsules during cooking of the cheese curd or to the nature of the fat capsules. Melting was not a concern in this study because the milk fat used had a melting point of 43°C, well above the highest temperature (38°C) attained during curd cooking. Fat capsules probably contain large pores through which the embedded enzymes can diffuse out into the whey. Braun and Olson (1986a) encapsulated haemoglobin in milk fat and reported no loss of the haemoglobin through diffusion at lower temperatures (below 20°C) but noted a rapid diffusion at temperatures above 32°C. They suggested that the rapid increase in haemoglobin diffusion was due to broken or leaky capsules. They (Braun and Olson, 1986a) however used normal milk butter (melting point 32°C) for encapsulation and it can not be said with certainty whether their observations holds true for the high melting milk fat fraction as used in this study.
3.9 CONCLUSION

The specific activity of enzyme encapsulated in selected gum have been studied. The results indicated that gellan and κ-carrageenan gums showed the highest specific enzyme activity of the encapsulated enzyme. Thus, enzyme encapsulated in gellan and κ-carrageenan gums retained more activity than for enzyme retained in alginate, agar, or κ-carrageenan-lbg. Enzyme encapsulated in agar gum showed the lowest activity. Enzyme activity appeared to have been influenced by both the immobilisation process and the nature of the gum used.

This first part of the study showed that gums could be employed for encapsulating enzymes. Thus it appeared feasible to use gum encapsulated enzymes in food processing as long as the gum capsules meet certain requirements. These requirements may be stability for instance during continuous fermentation or breakdown when the enzyme have to be released. The latter case is more relevant when the encapsulated enzyme is to be applied to enhance cheese ripening. Thus, in the second part of this study, gum encapsulated enzyme were used in cheese making with the aim of accelerating ripening. Two gums, gellan and κ-carrageenan, were selected for the trial based on their relatively high specific activity of the enzyme encapsulated in them. κ-Carrageenan was also selected due to its relative instability hence greater enzyme release potential during treatments in acidified or pH modified solutions as observed in the earlier study (Chapter 2).

The retention of enzyme capsules in cheese curd and the losses of enzymes in cheese whey were studied in the second study. All encapsulants, gellan, κ-carrageenan gums and milk fat exhibited very high entrapment levels in cheese curd. The extent of entrapment were however higher for the gum capsules, 90.0 and 91.2 % for κ-carrageenan and gellan respectively, than the milk fat capsules (73.5%). Entrapment rates were apparently related to the capsule sizes and encapsulant-protein interactions. Enzyme losses into whey were also lower for the gums than the milk fat. Gellan gum capsules treated cheeses gave the lowest enzyme losses (5.62%) while milk fat capsules showed the highest losses (17.93%). Enzyme losses into whey were attributed
to the stability of the capsules towards heating and agitation during cheese manufacture.

Findings from this study were very comparable to those conducted with liposomes which have been used for encapsulating enzymes (Kirby and Law, 1987) and such liposomes have since been successfully used in accelerating cheese ripening (Kirby et al., 1987). Thus, gellan, κ-carrageenan and hard milk fat fraction, just like liposome, have shown that they could also be used to encapsulate enzyme for accelerating cheese ripening.

Gellan and κ-carrageenan gums have shown in the second part of this study that they are potential enzyme encapsulant for food application. They have shown some extent of disintegration under conditions (pH and acidity) simulating those during cheese manufacture; retain reasonable activity for enzyme encapsulated in them; have good retention in cheese curd and help reduce premature loss of enzyme in cheese whey. These are the characteristics of a perfect enzyme encapsulant destined for cheese application. It would therefore be very interesting to find out if they can produce the results in cheese. Thus, in the next chapter, cheeses to which gum capsules and milk fat capsules were applied were tested for signs of accelerated cheese ripening.
CHAPTER 4

EVALUATING PROPERTIES OF THE ACCELERATED RIPENED CHEESE

4.1 SUMMARY

Degradation of casein due to proteolysis in ripening cheese was monitored by High Performance Capillary Electrophoresis (HPCE) method. Release of free amino acids in ripening cheeses was determined by reacting them with Trinitrobenzenesulphonic (TNBS) acid. Cheeses treated with enzyme capsules, showed higher rates of proteolysis than the untreated (control) cheese at most stages during ripening. Rate of proteolysis was greatest in cheeses treated with κ-carrageenan encapsulated enzymes. The addition of enzyme capsules to cheese had no significant effect on the microbial growth in cheese. Similarly, cheese texture and sensory quality of the ripened cheeses were not influenced by the addition of either gum or milk fat capsules. The activity of the applied encapsulated enzymes caused some changes in the textural and in certain sensory characteristics of the ripened experimental cheeses as compared to the untreated cheese. Textural and sensory scores were lower in cheeses that experienced a lot of proteolysis. Thus, incorporation of gellan, κ-carrageenan and milk fat enzyme capsules into cheese led to increased level of protein degradation which makes the technique potentially suitable for application as a mean of accelerating cheese ripening.
4.2 INTRODUCTION

Many studies have attempted to measure and predict cheese maturation by monitoring proteolysis and determining objective chemical parameters. Proteolysis is generally recognised as an acceptable indicator of cheese ageing with the concentration of free amino acids and amines correlating significantly with flavour development (Puchades et al., 1989). Methods describing the use of gel and paper electrophoresis (McSweeney et al., 1993), gel filtration, ion-exchange and reversed-phase chromatography (Bican and Spahni, 1993), and chemical analysis (Polychroniadou, 1988) have been reported.

A relatively new analytical method, capillary electrophoresis (CE) has recently been introduced as a separation technique with diverse application range. This method has been used for investigations on milk proteins and peptides (Chen and Zang, 1992; Otte et al., 1994) and produced promising results. The use of CE for monitoring proteolysis in cheeses has been reported recently by certain workers (Kristiansen et al., 1994; Weimer et al., 1996; Otte et al., 1996). These workers reached a similar conclusion, that is, CE method allows changes in casein degradation and peptide production during cheese ripening to be monitored and quantified in a single analysis. Capillary electrophoresis is a fast and simple electrophoretic technique that allows on-column detection of proteins and peptides at various wave lengths and data processing by computerised systems. Very small sample and buffer volumes are required and the use of chemicals mainly involves harmless substances (Li, 1992).

This investigation was carried out to study the changes during ripening of Cheddar cheese prepared from milk to which was added encapsulated commercial enzymes (Flavourzyme) in comparison to a control cheese made from milk without enzyme addition. The CE method was employed in this study as the principal method to monitor proteolysis in cheese. Because the CE technique is at its infancy in proteolysis monitoring, it was considered important to employ one other previously used methods as a check-measure. The chemical method using trinitrobenzenesulphonic (TNBS) acid was chosen because of its simplicity and good
reproducibility (Polychroniadou, 1988). Since its introduction by Satake et al (1960), the TNBS method has been used widely for the determination of free amino groups of proteins and protein hydrolysates (Ardo and Melsel, 1991). Unlike in the other methods of proteolysis determination, TNBS method does not require the elimination of protein from cheese extract (Polychroniadou, 1988). The assay is sensitive because TNBS reacts with α-amino groups to form products with high absorbance values, making possible the monitoring of every amino group released by the hydrolysis of a peptide bond (Polychroniadou, 1988). The method was also deemed appropriate for this study because the enzyme used, Flavourzyme, contains both endo- and exopeptidase activities (NOVO) which bring about peptide bond cleavage with the release of free amino groups (Ardo and Melsel, 1991).

The growth of micro-organisms during cheese maturation, the rheological and sensory properties of the cheese at the end of the maturation period were also evaluated.
4.3 MATERIALS AND METHODS

4.3.1 Materials
Trinitrobenzenesulfonic (TNBS) acid (analytical grade), D,L-Diothiothreitol, Zephiran and casein standards (α-, β, and κ-caseins) were obtained from Sigma Chemicals (Sydney, Australia). 0.1M Phosphate (pH 7.3) buffer and urea were of high performance capillary electrophoresis (HPCE) quality and supplied by Sigma Chemicals (Sydney, Australia). Plate count agar were from Oxoid (Sydney, Australia). All the other reagents were of analytical grade.

4.3.2 Composition of Cheeses

The compositional indices, that is, moisture, fat and salt contents for trial and control cheeses were determined after removal from the press.

The moisture content of the cheeses was determined by the oven-drying method (AOAC, 1990). 3.0 g samples of grated cheeses were weighed to the nearest 0.001g in pre-weighed dry dishes and lids containing sand. The dishes were transferred to an oven at 110°C and left for 24 h. After drying, the dishes were covered by their lids and removed from oven and placed in a desiccator and allowed to cool for 30 min. to room temperature. The cooled dishes were weighed to the nearest 0.001g. The determinations were done in duplicate. Moisture content was determined as follows:

\[
\text{Moisture content \%} = \frac{(\text{Weight of sample}) - (\text{Weight of dry sample} + \text{dish} + \text{lid})}{(\text{Weight of sample} + \text{dish} + \text{lid})} \times 100
\]

Cheese fat content was determined by the Babcock fat test method (Bartels et al., 1987b). 9.0 g of finely grated cheese samples were weighed to the nearest 0.01 g into a Babcock cream flask. 9.0 mL of hot water was added and the flask swirled. 0.5 ml of Zephiran, a quaternary ammonium compound, was added and the flask content again swirled. Concentrated sulphuric acid was added 2.0 mL at a time with intermittent swirling until a dark purple-black colour was obtained. The flask was
then centrifuged (1500 rpm) for 5 min. Hot water (65°C) was added to reach the shoulder of the flask and centrifuged again for 2 min. Hot water was again added until the liquid level reached about 2 cm from top of the flask. The flask and its content was further centrifuged for 1 min. and the % fat content read. Every cheese sample was determined in duplicate.

The salt (NaCl) content of the cheeses was determined by the Volhard method as described by Bartels et al (1987a). 2.0g of cheese sample was weighed to the nearest 0.001g into a pre-weighed 50 mL beaker. 20.0 mL of warm (55°C) water was added and the sample made into a slurry with the aid of a stirring rod. The slurry was transferred into a 250 mL Erlenmeyer flask. Exactly 25.0 mL of 0.1 N silver nitrate solution was added to the sample after which 10.0 mL of nitric acid and 50.0 mL of water was added. One clean boiling chip was added and the solution boiled on a hot plate. 15.0 mL of potassium permanganate was added in 5 ml portion and boiling continued for about 5 min. Boiling was continued until the brown colour disappeared. The hot solution was filtered through folded filter paper (Whatman #3) into a clean 250 mL Erlenmeyer flask. The filter paper was washed with water and the solution cooled to room temperature. 2.0 mL of ferric ammonium sulphate indicator was added and excess silver nitrate was titrated with 0.1 N potassium thiocyanate. Similarly, a blank was determined using 2.0 mL of water instead of the 2.0 g cheese sample. Two determinations were made for each cheese sample. Salt % was then calculated from the following equations:

\[
\text{Sodium chloride (Salt) \%} = \frac{[(\text{mL} \times N \text{AgNO}_3) - \text{mL} \times N \text{KSCN})] \times 0.0585}{\text{Weight of sample}} \times 100
\]

4.3.3 Determination of Proteolysis in Cheese

Ripening cheese were sampled after 1 day and after 2, 4, 8, 12 and 16 weeks and enzymatic breakdown of caseins monitored using capillary electrophoresis method.
Free amino groups produced as a result of enzymatic protein breakdown were determined by reacting them with trinitrobenzenesulfonic acid.

4.3.4 Suspending Cheese and Isolating Casein

Cheese was suspended in a buffer solution by the method of Gripon et al (1975). Ten g of cheese was suspended in 40 mL of 0.5 M sodium citrate buffer (pH 7.0) in a 500 ml beaker placed in a water bath at 40 °C. It was stirred (2000 rpm) twice for 30s with an impeller stirrer and cooled to 22 °C. The volume of the cheese suspension was then adjusted to 200 ml with deionised water (Millipore).

Whole casein from the cheese suspension was isolated by the method described by McKenzie (1971) as follows. To 100 ml of the cheese suspension was added 1 M HCl slowly from a burette whose tip was kept below the surface of the suspension at 20 °C. Addition of the HCl was continued with magnetic stirring over a period of 40-45 min until a pH value of 4.5-4.6 was reached. Stirring was continued for a further 30 min. The precipitate was sedimented at 2000 rpm in a centrifuge (Selecta P, Italy). It was resuspended, washed twice with 40 ml distilled water (pH 4.6, adjusted using 0.1M HCl) and centrifuged as previously described. The isolated wet casein was cooled in the freezer (-14 °C) for 1 hour and freeze dried (Dynavac Freeze Drying Unit) for 24 h prior to analyses.

4.3.5 Instrumentation

Capillary electrophoresis separations were performed using a Hewlett Packard 3DCE System G1600AX (Waldbornn, Germany) comprising of a capillary electrophoresis unit with built-in diode ray detector. An HP Chemstation installed in a PC-HP VectraXM2 4/100i computer (Hewlett Packard, Germany) was used for system control, data collection and data analysis. Untreated silica capillary column of 64.5 cm total length, 56.0 cm effective length, 50.0 μm internal diameter was used in the instrument cartridge. Peak identities were assigned by running external protein standards separately. Values of migration times and areas were calculated as relative to the external standards.
4.3.6 Capillary Electrophoresis

Sample solutions, sample and run buffers were prepared according to a modified method of Cattaneo et al (1996). Sample buffer was prepared by dissolving urea and D,L-dithiothreitol (DTT) in 100 ml of 0.1 M phosphate buffer (pH 7.0) to give concentrations of 9 M and 30 mM for urea and DTT respectively. DTT was used as an internal marker. The run buffer was made by dissolving only urea in 100 ml of 0.1 M phosphate buffer (pH 7.0) to give a concentration of 6 M. Sample solutions were prepared by dissolving 80 mg of the extracted casein powder or 10 mg pure casein (standard) in 10 ml of the sample buffer. The solution was centrifuged at 2000 rpm for 10 min and then filtered through a 0.45 μm filter before use. Standard curve was prepared by dissolving β-Casein standard in a sample buffer prepared without including urea to give concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/ml. The solutions were centrifuged (2000 rpm, 10 min) and filtered through a 0.45 μm filter before use.

Analyses were performed according to a modified method of Kristiansen et al (1994). Each standard and sample solution was injected hydrostatically for 15 s and separated at a constant voltage of 10 kV and current of 112 μA at 36 ºC for 25 min. UV detection was performed at 214 nm. After each separation, the capillary was sequentially flushed for 3s with 1.0 M sodium hydroxide and the run buffer. Standard and sample solutions were run through the HPCE in duplicate and results were expressed as concentration of β-casein in μg/mL.

4.3.7 Determination of Free Amino Groups

Proteolysis in the ripening cheese was monitored by measuring the free amino groups using the method proposed by Polychroniadou (1988) as follows: One g of cheese was dispersed in 20 ml 0.1 M sodium borate buffer (pH 9.5), warmed at 45 ºC for 15 min with stirring and centrifuged (Selecta P, Italy) at 4300 rpm for 20 min. A 6 ml portion of the supernatant was diluted to 100 ml with distilled water.
Five dilutions of glycine (used as standard amino acid) solutions was prepared in 0.1M HCl to give solutions of 0.01, 0.1, 1.0, 2.5, and 4.5 mg/mL concentrations and used for preparing the standard curve.

From the cheese extract and the standard solutions, a portion (0.5 ml) was added to 0.5 ml borate buffer and TNBS reagent (1 ml; 1 mg/ml) was added. After thorough mixing, the solution was incubated at 37 °C for 60 min. Blanks were prepared with 0.5 ml of water instead of cheese extract. The reaction was stopped by adding 2 ml of 0.1 M sodium phosphate containing 1.5 mM sodium sulphate and the absorbance was read at 420 nm on a spectrophotometer (Ultrospec-LKB Biochrom 000122, Sydney). Results were then read from the standard curve and expressed as mg/mL of amino acid with the assumption that the concentration of glycine was equal to the concentration of free amino groups. Analyses for cheese extract samples were made in triplicate and for standard solutions in duplicate.

4.3.8 Microbiology

The cheeses were sampled after 1 day and 8 weeks according to standard methods (International Dairy Federation, 1985) and microbiological analyses were made according to International Dairy Federation (1992). Briefly, cheese samples (10 g) were blended in 90 mL of 0.1% peptone water (pH 7.0) for 1 min in a Stomacher Laboratory Blender 400 (FSE, Sydney). It was then serially diluted up to $10^6$ in peptone water. One mL aliquots of the dilutions were added to sterile Petri dishes prepared in duplicate for each cheese sample. Fifteen mL of prepared plate count agar was added at 37 °C and the content mixed and the plates incubated at 30 °C. The total bacterial count was then made after 3 days of incubation.

4.3.9 Determination of Cheese Textural and Sensory properties

The textural and sensory properties of the trial cheeses were assessed at the stage of maturation when the determined level of casein degradation (HPCE method) was equivalent to that in a 6 month old cheese (untreated). In this study, the β-casein content of untreated 6 month old cheese was 1.39mg/ml as determined by the HPCE
method. This value was equivalent to the \( \beta \)-casein content in 5 month old enzyme treated cheeses (Table 4.2). Textural and sensory evaluations were made for the purpose of determining the effect of added gum gels and accelerated ripening on the final quality of cheeses produced.

### 4.3.10 Measurement of Cheese Texture Profile

Cheese curds were drawn and their textural properties measured according to the modified method of Raphaelides et al (1995). Four cubical samples (1.3 x 1.3 x 1.3 cm) were bored from each cheese block at different depths to limit the effects of surface drying (Jack et al., 1993). The samples were held at room temperature (21-24°C) for 1 h before testing. Each sample was compressed axially using a Texture Profile Analyser (TA, XT2, Version 5.15) to 50% of their original heights in two consecutive compression cycles by a 30 mm diameter flat crosshead. Other test conditions were: crosshead speed was 1.0 mm/s and contact speed was 5.0 g.

The textural parameters measured from the double curves were:

- **Hardness** - the peak force during first compression cycle.
- **Cohesiveness** - the ratio of positive area during second compression to that during the first compression.
- **Springiness** - the height that the food recovers during the time that elapsed between the end of the second cycle.
- **Gumminess** - the energy required to disintegrate a seem-solid food product to a state ready for swallowing; the product of hardness \( \times \) cohesiveness.
- **Chewiness** - the energy required to masticate a solid food product to a state ready for swallowing; the product of gumminess \( \times \) springiness.

(Szczcesniak, 1966; Bourne, 1978; Yang and Taranto, 1982).
4.3.11 Sensory Evaluation

The sensory panel composed of 32 members of the School of Food Science’s students and staff, 18-45 years of age, were untrained but most of them have had previous experience of sensory evaluation of food products including dairy products. The panel members were individually instructed and the vocabulary used in the questionnaire defined.

Each cheese sample was cut into 2 cm cubes. The test was carried out according to the procedure of Muir et al (1995). Four samples together with plain biscuit were presented to each panellist at room temperature in white disposable paper plates. The order of presentation was randomised for each panellist and between each of the three sessions. Panel members were asked to rate each cheese on a 12.5 cm undifferentiated scale with anchor points at each end and the rating was measured.

Sensory evaluation was conducted in sensory cubicles and assessors were instructed to consume the plain biscuit and to rinse their mouth with fresh, cold water before and between evaluation of samples. There was no time constraint on assessors. Assessors were offered chocolate pieces as a token of appreciation at the end of each session.

4.3.12 Statistical Analysis

Analysis of variance was employed using a computer statistical package, Costat, and the significant differences of means were tested by Duncan’s Multiple Range Test (Steel and Torrie, 1980).
4.4 RESULTS AND DISCUSSION

4.4.1 Effect of treating cheese with enzyme capsules on cheese composition

The composition of the control and trial cheeses are presented in Table 4.1. Moisture content ranged from 37.4-40.3%, fat (31.5-33.8%) and salt (1.67-1.89%). The moisture content of all gum capsules-treated cheeses were significantly higher (P<0.01) than that of control cheese. The moisture content of milk fat capsules-treated cheeses were however not significantly different from that of control cheeses. The moisture content values of gum capsules treated-cheeses were also above the maximum allowable moisture content (39%) in Cheddar cheese as stipulated by the Australian Standard (Anon, 1994).

This finding is similar to those of Manning et al (1986) and Kanombirira and Kailasapathy (1995) who found high moisture content in cheeses where gum was incorporated as compared to control cheese. The high moisture content of gum capsules-treated cheese curds was due to the hydrophilic nature of both carrageenan and gellan gums which retained moisture in cheese. The salt and fat contents of the trial cheeses were very comparable with those of control. The fat content of the milk fat capsules-treated cheeses were however significantly higher (P<0.05) than the rest of the trial and control cheeses. This was due to the extra fat added to the cheese as capsules. Thus, it may be concluded that, application of enzyme capsules had minimal effects on the fat and salt contents of the cheeses. Application of gum capsules however increased the moisture content of cheeses.
Table 4.1  Chemical Composition of Experimental Cheeses

<table>
<thead>
<tr>
<th>Substance</th>
<th>Value 1</th>
<th>Value 2</th>
<th>Value 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>12.3%</td>
<td>13.2%</td>
<td>10.5%</td>
</tr>
<tr>
<td>Fat</td>
<td>25.6%</td>
<td>23.4%</td>
<td>24.8%</td>
</tr>
<tr>
<td>Moisture</td>
<td>25.6%</td>
<td>25.4%</td>
<td>25.6%</td>
</tr>
<tr>
<td>Ash</td>
<td>1.2%</td>
<td>1.3%</td>
<td>1.1%</td>
</tr>
</tbody>
</table>

1 Data from experiments conducted in 2023.
<table>
<thead>
<tr>
<th>Cheese</th>
<th>Enzyme</th>
<th>Salt</th>
<th>Moisture</th>
<th>Fat</th>
<th>MNF</th>
<th>FDM</th>
<th>S/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.89a</td>
<td>37.6c</td>
<td>32.6b</td>
<td>55.8c</td>
<td>50.8c</td>
<td>5.0a</td>
<td></td>
</tr>
<tr>
<td>κ- Carrageenan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kl</td>
<td>1.88a</td>
<td>38.4b</td>
<td>31.8b</td>
<td>56.3b</td>
<td>51.6c</td>
<td>4.9a</td>
<td></td>
</tr>
<tr>
<td>Km</td>
<td>1.86a</td>
<td>38.7b</td>
<td>32.1b</td>
<td>57.0b</td>
<td>52.4b</td>
<td>4.8a</td>
<td></td>
</tr>
<tr>
<td>Kh</td>
<td>1.90a</td>
<td>38.2b</td>
<td>31.7b</td>
<td>56.0b</td>
<td>51.3c</td>
<td>4.9a</td>
<td></td>
</tr>
<tr>
<td>Gellan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gl</td>
<td>1.82a</td>
<td>39.7a</td>
<td>32.0b</td>
<td>58.4a</td>
<td>53.1b</td>
<td>4.6a</td>
<td></td>
</tr>
<tr>
<td>Gm</td>
<td>1.87a</td>
<td>40.3a</td>
<td>31.5b</td>
<td>58.8a</td>
<td>52.8b</td>
<td>4.6a</td>
<td></td>
</tr>
<tr>
<td>Gh</td>
<td>1.91a</td>
<td>39.9a</td>
<td>31.8b</td>
<td>58.5a</td>
<td>52.9b</td>
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<td></td>
</tr>
<tr>
<td>Milk fat</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Fl</td>
<td>1.80a</td>
<td>37.8c</td>
<td>33.0a</td>
<td>56.4b</td>
<td>53.1b</td>
<td>4.8a</td>
<td></td>
</tr>
<tr>
<td>Fm</td>
<td>1.89a</td>
<td>37.5c</td>
<td>33.4a</td>
<td>56.3b</td>
<td>53.4b</td>
<td>5.0a</td>
<td></td>
</tr>
<tr>
<td>Fh</td>
<td>1.87a</td>
<td>37.6c</td>
<td>33.8a</td>
<td>56.8b</td>
<td>54.2a</td>
<td>5.0a</td>
<td></td>
</tr>
</tbody>
</table>

1Means of six determinations
2C - Control; K - κ-Carrageenan; G - Gellan; F - Milk fat; l, m and h are low, medium and high levels respectively of enzyme applications.
3, 5, 6, 7, 8Means within a column followed by a different letter were different significantly (P<0.05)
4Means within the column followed by a different letter were significantly different (P<0.01)
5MNFS = Moisture(%) x 100
   (100 - Fat %)
7FDM = Fat % x 100
   (100 - Moisture %)
8S/M = Salt % x 100
   Moisture %
4.4.2 Effect of treating cheese with enzyme capsules on casein degradation during ripening as determined by the HPCE method

Most of the $\alpha_s$-casein in milk was broken down during the cheese making process (Appendix 5). This left $\beta$-casein as the major component of milk proteins in the cheese curd. This observation was in agreement with those of (McSweeney et al., 1993a) who indicated that chymosin substantially breaks down $\alpha_s$-casein during the early stage of cheese manufacture. Thus in this study, proteolysis during cheese ripening was assessed as the degradation of $\beta$-casein by the HPCE method. The observed degradation of $\beta$-casein was used as a basis for determining the extent and rate of acceleration of cheese ripening.

The $\beta$-casein contents of the cheeses are presented in Table 4.2. Cheeses were sampled after various time intervals during ripening and their protein separated by HPCE. The area under $\beta$-casein peak was measured and the $\beta$-casein content calculated relative to the external standard by the HP Chemstation (Hewlett Packard, Germany) software (Appendix 4). There was a marked reduction in $\beta$-casein content in all cheese treatments after the 5 months storage period. However, while all $\kappa$-carrageenan capsules treated cheeses and cheeses treated with gellan and milk fat capsules at high level enzyme content showed significant differences ($P < 0.05$) in $\beta$-casein content compared to control cheeses after 5 months, treatments using either gellan or milk fat capsules at both low and medium level enzyme content were not significantly different. In general, cheeses treated with capsules containing larger concentrations of enzyme contained progressively lesser $\beta$-casein right from after one day ripening and through out the 5 month period of ripening. The observed values of $\beta$-casein content for all the treatments after 5 month of ripening were however all below that of a 6 month old untreated cheese (1.39 mg/ml) prepared in the conventional way.
Table 4.2. Changes in β-casein content of ripening cheeses by HPCE Method
<table>
<thead>
<tr>
<th>Treatment</th>
<th>1 Day</th>
<th>1 Month</th>
<th>3 Months</th>
<th>5 Months</th>
<th>6 Months</th>
<th>Casein Remaining after 5 months(%)&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.34</td>
<td>1.83</td>
<td>1.64</td>
<td>1.42</td>
<td>1.39</td>
<td>60.68a</td>
</tr>
<tr>
<td>K&lt;sub&gt;l&lt;/sub&gt;</td>
<td>2.32</td>
<td>1.69</td>
<td>1.55</td>
<td>0.79</td>
<td>---</td>
<td>34.05d</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>2.22</td>
<td>1.62</td>
<td>1.43</td>
<td>0.65</td>
<td>---</td>
<td>29.28e</td>
</tr>
<tr>
<td>K&lt;sub&gt;h&lt;/sub&gt;</td>
<td>2.11</td>
<td>1.48</td>
<td>1.26</td>
<td>0.52</td>
<td>---</td>
<td>24.65e</td>
</tr>
<tr>
<td>G&lt;sub&gt;l&lt;/sub&gt;</td>
<td>2.49</td>
<td>1.87</td>
<td>1.77</td>
<td>1.34</td>
<td>---</td>
<td>53.82b</td>
</tr>
<tr>
<td>G&lt;sub&gt;m&lt;/sub&gt;</td>
<td>2.38</td>
<td>1.92</td>
<td>1.57</td>
<td>1.24</td>
<td>---</td>
<td>52.10b</td>
</tr>
<tr>
<td>G&lt;sub&gt;h&lt;/sub&gt;</td>
<td>2.32</td>
<td>1.73</td>
<td>1.48</td>
<td>1.05</td>
<td>---</td>
<td>45.26c</td>
</tr>
<tr>
<td>F&lt;sub&gt;i&lt;/sub&gt;</td>
<td>2.3</td>
<td>1.96</td>
<td>1.84</td>
<td>1.34</td>
<td>---</td>
<td>58.26a</td>
</tr>
<tr>
<td>F&lt;sub&gt;m&lt;/sub&gt;</td>
<td>2.19</td>
<td>1.91</td>
<td>1.81</td>
<td>1.25</td>
<td>---</td>
<td>57.08a</td>
</tr>
<tr>
<td>F&lt;sub&gt;h&lt;/sub&gt;</td>
<td>2.16</td>
<td>1.85</td>
<td>1.78</td>
<td>1.08</td>
<td>---</td>
<td>50.00b</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means of duplicate determinations.

<sup>2</sup>C - Control; K- k-Carrageenan; G- Gellan; F- Milk fat; l, m and h are levels low, medium and high respectively of enzyme applications as in Chapter 4.

<sup>3</sup>Means within a column followed by a different letter were different significantly (P<0.05).
The rate of β-casein breakdown throughout the 5 month period of observation for the treated cheeses is shown in Figure 4.1. The percentage of β-casein remaining, expressed as percentage of the amount present in control cheese immediately after pressing (i.e., after less than one day or zero week), for all treatment showed similar trends of decrease as ripening progressed (Table 4.2). Cheese treated with κ-carrageenan encapsulated enzyme at the highest level of flavourzyme content showed higher rate of β-casein breakdown than control cheese throughout the 5 month period. The other cheese treatments showed varied trends of β-casein breakdown when compared to the control cheese. However, all treatments had lower levels of β-casein remaining after 5 months when compared with the control cheese. Control cheese had 60.68% β-casein remaining after 5 months while the treated cheeses had 24.65 to 58.26% of β-casein remaining. This finding is consistent with those of Kirby et al (1987) who while studying cheese ripening acceleration using liposomes-encapsulated enzymes reported that 68% of the β-casein remained intact in the untreated cheese after three months of ripening. Law and Wigmore (1982) also noted a progressive decrease in β-casein levels in enzyme treated cheeses after 2 months ripening and a reduction in the amount of β-casein of approximately 60% in treated cheeses after 4 months.
Figure 4.1 \( \beta \)-Casein degradation in ripening cheeses

Legends: C is control cheese;
- KL, KM, and KH are cheeses containing low, medium and high concentration respectively \( \kappa \)-carrageenan encapsulated enzyme;
- GL, GM, and GH are cheeses containing low, medium and high concentration respectively gellan encapsulated enzyme;
- FL, FM, and FH are cheeses containing low, medium and high concentration respectively milk fat encapsulated enzyme;
The higher rate of β-casein reduction in the κ-carrageenan capsules treated cheeses reflected the destabilisation observed (Chapter 2) when κ-carrageenan gels were treated in acidified or pH modified solution. Thus lactate in the cheese played a role in the release of enzyme from κ-carrageenan capsules by chelating calcium and disrupting the capsules (Roy et al., 1987). Interaction that normally occurs between κ-carrageenan and casein (Snoeren et al., 1975) must have also influenced the rate of β-casein breakdown in cheese. This interaction, especially those between β-casein and carrageenan with Ca$^{2+}$ as a cross-linker (Dalgleish and Morris, 1988), bring the substrate (casein) into closer proximity to the enzyme which is entrapped in the carrageenan gel. This enhances the chance for the encapsulated enzyme to interact with casein hence the observed higher rate of β-casein degradation. It can thus be said that the interaction between casein and carrageenan has both minimal effect on casein conformation or poses no steric hindrance on casein molecular structure. Thus enzyme-casein coupling occurs even when the casein has already interacted with carrageenan. This is in conformation with the views of Oakenfull et al., (1996) who indicated that interaction between casein and carrageenan does not affect their individual molecular behaviour. This is because casein micelles simply adsorb κ-carrageenan leaving either the casein or the κ-carrageenan free to participate in their individual reactions. For instant, the κ-carrageenan can still undergo the coil-to-helix transition and form a gel network.

The observed slower rate of β-casein degradation in cheeses treated with gellan capsules suggests that these capsules have probably been releasing their enzyme contents very slowly. This implies that gellan capsules remain relatively stable within the cheese curd. This was consistent with the earlier observed stability of gellan gels under acidic and pH conditions similar to those encountered during cheese making (Chapter 2). Stability of the gellan capsules could be attributed to its ability to form gels with a wide variety of ions (Grasdalen and Smidsrod, 1987) besides Ca$^{2+}$. Thus, even if lactic acid in cheese co-ordinated Ca$^{2+}$ in gellan capsules, the capsules maintained their integrity due to interaction with other ions present in the complex cheese curd system. Gellan gum interaction with casein under normal conditions of acidity has not been confirmed (Sanderson and Clarke, 1983). So unlike for
carrageenan which interacts with casein, there is a lower chance for casein to come close to gellan encapsulated enzyme. This contributed to the lower rate of β-casein degradation as compared to carrageenan encapsulated enzyme.

Milk fat capsules seems to have been stable because cheese treated with them showed a slow degradation of β-casein. Magee et al (1981) had suggested that the activity of lipase enzyme on the milk fat capsules facilitates enzyme release from milk fat capsules. This mechanism was probably very slow in this experiment to bring about a significant earlier reduction in β-casein contents. Heat is another means by which milk fat encapsulated enzyme is released when the milk fat melts. Heat was not effective in this study because the milk fat used in the preparation of capsules in this experiment had a higher melting point (43°C) than the highest temperature (38°C) applied during cheese production. The slower rate of degradation of β-casein in milk fat capsules treated cheeses could also be attributed to the relatively higher loss (~25%) of the added enzymes into cheese whey (Chapter 3).

4.4.3 Effect of adding enzyme capsules on proteolysis in Cheese determined by TNBS method

The results for the determination of free amino group, in cheeses sampled at different time intervals, by the TNBS method are in Table 4.3 and Fig 4.2. The results indicate that the free amino group content of the cheeses were significantly increased by the addition of enzyme capsules as compared to the control (untreated) cheese. The influence of enzyme capsules addition on cheese proteolysis was significant within 2 weeks (P < 0.05) and increased during ripening. There was significantly (P< 0.05) higher levels of free amino groups in treated cheeses as compared to the control cheese and the difference increased during further ripening.
Table 4.3. Changes in the content of free amino group in ripening cheese by TNBS method
<table>
<thead>
<tr>
<th>Age (days)</th>
<th>C</th>
<th>Kl</th>
<th>Km</th>
<th>Kh</th>
<th>Gl</th>
<th>Gm</th>
<th>Gh</th>
<th>Fl</th>
<th>Fm</th>
<th>Fh</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.055d</td>
<td>0.026d</td>
<td>0.068d</td>
<td>0.072d</td>
<td>0.047d</td>
<td>0.055d</td>
<td>0.302e</td>
<td>0.421e</td>
<td>0.622e</td>
<td>0.681d</td>
</tr>
<tr>
<td>14</td>
<td>0.695c</td>
<td>1.364c</td>
<td>1.496c</td>
<td>1.262c</td>
<td>1.1c</td>
<td>1.382c</td>
<td>1.276d</td>
<td>0.664d</td>
<td>0.727d</td>
<td>0.8d</td>
</tr>
<tr>
<td>30</td>
<td>0.708b</td>
<td>1.454b</td>
<td>1.682b</td>
<td>3.187b</td>
<td>1.171c</td>
<td>1.524b</td>
<td>1.584c</td>
<td>0.795c</td>
<td>1.041c</td>
<td>1.176c</td>
</tr>
<tr>
<td>60</td>
<td>0.821b</td>
<td>1.550b</td>
<td>1.794b</td>
<td>3.306b</td>
<td>1.242b</td>
<td>1.671b</td>
<td>1.758b</td>
<td>0.941c</td>
<td>1.273b</td>
<td>1.368b</td>
</tr>
<tr>
<td>90</td>
<td>0.972b</td>
<td>1.676b</td>
<td>1.805b</td>
<td>3.57a</td>
<td>1.351b</td>
<td>1.738a</td>
<td>1.947b</td>
<td>1.266b</td>
<td>1.385b</td>
<td>1.574a</td>
</tr>
<tr>
<td>120</td>
<td>1.139a</td>
<td>1.794a</td>
<td>2.013a</td>
<td>3.641a</td>
<td>1.402a</td>
<td>1.815a</td>
<td>2.198a</td>
<td>1.301a</td>
<td>1.502a</td>
<td>1.639a</td>
</tr>
<tr>
<td>150</td>
<td>1.286a</td>
<td>1.983a</td>
<td>2.172a</td>
<td>3.686a</td>
<td>1.497a</td>
<td>1.873a</td>
<td>2.233a</td>
<td>1.39a</td>
<td>1.567a</td>
<td>1.715a</td>
</tr>
</tbody>
</table>

\(^1\)Means of triplicate determinations; means within a column followed by a different superscript were different significantly (P < 0.05).

C - Control; K - κ-Carrageenan; G - Gellan; F - Milk fat; l, m and h are levels low, medium and high respectively of enzyme applications as in Chapter 4.
Cheeses treated with κ-carrageenan capsules showed a high rate of increase in free amino groups during the first month of ripening before slowing and dropping to a similar rate as those of gellan and milk fat capsules treatments. The increase in free amino groups parallels the β-casein breakdown as determined by the HPCE method (Fig 4.1). This was expected because amino groups are produced in cheese as a consequence of protein breakdown during ripening (Fox et al, 1993), thus an increase in free amino groups in treated cheese over that of control at any given time during ripening indicates an acceleration of ripening. Kuchroo et al (1983) and Lemieux et al (1990) showed that the concentration of free amino acids generally increased with cheese ripening time by TNBS method.
Figure 4.2 Proteolysis during the ripening of cheeses

Legends: C is control cheese;
KL, KM, and KH are cheeses containing low, medium and high concentration respectively κ-carrageenan encapsulated enzyme;
GL, GM, and GH are cheeses containing low, medium and high concentration respectively gellan encapsulated enzyme;
FL, FM, and FH are cheeses containing low, medium and high concentration respectively milk fat encapsulated enzyme;
Capsules with higher enzyme content exhibited higher amino groups production under almost all treatments (Figure 4.2). This indicates that enzyme release took place in cheese during ripening. The observed differences in free amino group content of the cheeses upon removal from the press, that is at day 0, (Table 4.3) may be attributed to the rupture of capsules during curd pressing. The released enzyme from ruptured capsules could have acted upon the proteins during the 16 h of curd pressing. Differences in capsule materials and levels of enzyme applications was probably responsible for the different TNBS values.

4.4.4 Total bacteriological count in ripening cheeses

Micro-organisms contribute significantly towards determining the quality of ripening cheese. For instance, McSweeney et al (1993b) found higher concentration of free amino acids in cheeses with higher non-starter lactic acid bacteria. Thus it is important to assess the effect of adding enzyme capsules to cheese on the growth of micro-organisms in cheese.

The counts of total bacterial growth in the cheeses are indicated in Table 4.4. Total bacterial counts ranged between 32-52 x 10^6 on day one to 65-75 x 10^6 after 4 weeks and dropped to 34-56 x 10^6 after 12 weeks. These values are in conformity to those reported by Cromie et al (1987). Trial cheeses had higher but in most cases not significant (P < 0.05) total bacterial count than the control cheese.
Table 4.4. Total bacterial count in ripening cheeses
<table>
<thead>
<tr>
<th>Cheese Age (Days)</th>
<th>Control</th>
<th>Kl</th>
<th>Km</th>
<th>Kh</th>
<th>Gl</th>
<th>Gm</th>
<th>Gh</th>
<th>Fl</th>
<th>Fm</th>
<th>Fh</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32a</td>
<td>43a</td>
<td>45a</td>
<td>40a</td>
<td>42a</td>
<td>38a</td>
<td>48ab</td>
<td>44a</td>
<td>38a</td>
<td>52b</td>
</tr>
<tr>
<td>30</td>
<td>68a</td>
<td>69a</td>
<td>72a</td>
<td>68a</td>
<td>75a</td>
<td>67a</td>
<td>73a</td>
<td>70a</td>
<td>65a</td>
<td>76a</td>
</tr>
<tr>
<td>60</td>
<td>34a</td>
<td>40a</td>
<td>47ab</td>
<td>48ab</td>
<td>46a</td>
<td>40a</td>
<td>56b</td>
<td>43a</td>
<td>51b</td>
<td>34a</td>
</tr>
</tbody>
</table>

1 Means of 3 replications. Means within an age grouping (row) followed by a common letter are not significantly different (P > 0.05)

K - κ-Carrageenan; G - Gellan; F - Milk fat; l, m, and h are low, medium and high levels of enzyme applications as in Chapter 4.
The observed higher counts in trial cheeses over the control cheese probably resulted from bacteria introduced with the capsules slightly contaminated during washing prior to addition to cheese milk. However, because of the nature of the capsules used in this study, complex carbohydrate (gum) and fat and the rather strong salt solution used during capsules preparation, microbial growth was expected to be minimal. Thus addition of enzyme capsules to cheese coupled with a high standard of hygiene should not significantly influence bacterial growth in cheese. The increase in total count observed in all cheeses after 30 days must have been due to growth of the starter bacteria and non-starter lactic acid bacteria. Cromie et al (1987) indicated that lactobacilli increase in number rapidly during the first 4 weeks of cheese storage. The decrease in total count after 30 days probably reflects a reduction in the number of viable starter cells during this period (Folkertsma et al., 1996).

The increase in total bacterial count during the first 30 days (Table 4.4) could have also contributed to the observed increase in amino acid concentration in cheese within this period of ripening (Section 4.4.2). Non-lactic acid bacteria are known to significantly contribute to the production of free amino acids in ripening cheese (McSweeney et al (1993b).

4.4.5 The influence of cheese composition and adding gum and milk fat enzyme capsules on ripened cheese quality

4.4.5.1 Influence on Cheese Texture

The cheese textural parameters measured are presented in Table 4.5. There were significant differences between the experimental and control cheeses for all the textural parameters i.e. springiness, cohesiveness, gumminess, hardness and chewiness between the trials and the control cheeses. There was reduction in mean score of most textural parameters when κ-carrageenan capsules of higher enzyme concentrations were used. As shown in Figure 4.3, the mean scores for gumminess, hardness and chewiness were significantly (P <0.01) higher for control cheese than for the trial cheeses. This finding was in agreement with those of Alkhalaf et al (1988) who also found a reduction in hardness, gumminess and chewiness in Saint
Paulin cheeses treated with free or liposome encapsulated Neutrase. Cheeses where κ-carrageenan enzyme capsules were applied received the lowest mean score for gumminess, hardness and chewiness.
Table 4.5. Changes in textural properties of 5 month ripened experimental cheeses
<table>
<thead>
<tr>
<th>Sample</th>
<th>Textural Properties&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Springiness</td>
</tr>
<tr>
<td></td>
<td>(x 10&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Control&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.79a</td>
</tr>
<tr>
<td>Kl</td>
<td>0.61b</td>
</tr>
<tr>
<td>Km</td>
<td>0.48c</td>
</tr>
<tr>
<td>Kh</td>
<td>0.27d</td>
</tr>
<tr>
<td>Gl</td>
<td>0.79a</td>
</tr>
<tr>
<td>Gm</td>
<td>0.76a</td>
</tr>
<tr>
<td>Gh</td>
<td>0.61b</td>
</tr>
<tr>
<td>Fl</td>
<td>0.74a</td>
</tr>
<tr>
<td>Fm</td>
<td>0.65b</td>
</tr>
<tr>
<td>Fh</td>
<td>0.78a</td>
</tr>
</tbody>
</table>

<sup>*</sup>Means of four measurements; Values followed by the same letter are not significantly different at $P < 0.05$.

<sup>2</sup>Six months ripened untreated cheese.
The reduction in gumminess, hardness and chewiness in trial cheeses was most likely due to the degradation of casein as a result of the activities of the enzymes released from the capsules. Casein is largely responsible for forming the cheese structure. Thus, the low score noted for κ-carrageenan was expected since cheeses treated with enzymes encapsulated in κ-carrageenan exhibited the highest level of β-casein degradation in the earlier study (Table 4.2).

The changes in the treated cheese texture are consistent with the values of MNFS and S/M of these cheeses (Table 4.1). Most of the treated cheeses had higher MNFS value and lower S/M values than the control cheeses. High MNFS values and lower S/M values bring about a high rate of casein breakdown in cheeses (Lawrence et al., 1987) hence the low scores for texture parameters as obtained in treated cheeses. However, the values of MNFS and S/M for κ-carrageenan treated cheeses were generally lower and higher respectively than those of gellan treated cheeses. This is in spite of the fact that κ-carrageenan treated cheeses showed higher proteolysis than the gellan treated cheeses (Table 4.2). This observation therefore implies that, although MNFS and S/M values are important in determining rates of proteolysis in maturing cheeses, other factors are equally critical. One such factor, with respect to this study, must have been the extent of capsule disintegration in the curd. In Chapter 2 of this thesis it was observed that κ-carrageenan capsules had better ability to release enzymes than gellan capsules. Consequently, κ-carrageenan capsules-treated cheeses had higher levels of proteolysis than gellan capsules-treated ones.
Figure 4.3 Textural characteristics of the ripened cheeses

Legends: C is control cheese;
KL, KM, and KH are cheeses containing low, medium and high concentration respectively κ-carrageenan encapsulated enzyme;
GL, GM, and GH are cheeses containing low, medium and high concentration respectively gellan encapsulated enzyme;
FL, FM, and FH are cheeses containing low, medium and high concentration respectively milk fat encapsulated enzyme;
Although use of enzyme capsules resulted in higher rate of protein breakdown in cheese hence accelerated ripening, the lower mean score for textural parameters of the experimental cheeses as compared to the control cheese (Figure 4.3) may present a problem with product identity and acceptance. The lower mean score for textural parameters for trial cheeses could have also been as a result of moisture retention in gum treated cheese (Table 4.1). Excessive moisture retention in cheese during manufacture is known to result in soft and crumbly texture in finished cheese (Manning, 1985).

4.4.5.2 Influence on Sensory quality

The results of the sensory evaluation of ripened cheeses are shown in Table 4.6. The untreated control cheese scored highest for appearance but was not significantly different from the scores of cheeses treated with GI and Fh capsules. Cheeses treated with κ-carrageenan capsules (KI, Km and Kh) gained the lowest score for appearance. The lower scores for the cheeses treated with κ-carrageenan capsules must have been due to the higher level of proteolysis in the cheese as compared to the other trial cheeses which led to a crumbly and less cohesive texture. Excessive proteolysis in cheese was considered (Law and Wigmore, 1982) to lead to low score in the textural properties of the cheese.
Table 4.6. Sensory scores of 5 months ripened experimental cheeses
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Appearance</th>
<th>Flavour</th>
<th>Aroma</th>
<th>Texture</th>
<th>Bitter Aftertaste</th>
<th>Overall Acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control¹</td>
<td>8.48a</td>
<td>6.18a</td>
<td>6.19a</td>
<td>5.06ab</td>
<td>5.11c</td>
<td>7.05a</td>
</tr>
<tr>
<td>Kl</td>
<td>3.74d</td>
<td>7.13a</td>
<td>5.51a</td>
<td>4.83ab</td>
<td>9.55a</td>
<td>3.26b</td>
</tr>
<tr>
<td>Km</td>
<td>3.29d</td>
<td>6.98a</td>
<td>5.23a</td>
<td>4.30ab</td>
<td>9.49a</td>
<td>3.04cb</td>
</tr>
<tr>
<td>Kh</td>
<td>2.51e</td>
<td>7.54a</td>
<td>3.47b</td>
<td>2.79c</td>
<td>9.87a</td>
<td>1.18c</td>
</tr>
<tr>
<td>G1</td>
<td>7.09ab</td>
<td>6.67a</td>
<td>6.59a</td>
<td>4.11b</td>
<td>4.92c</td>
<td>7.34a</td>
</tr>
<tr>
<td>Gm</td>
<td>5.70bc</td>
<td>6.46a</td>
<td>6.84a</td>
<td>5.25ab</td>
<td>5.81c</td>
<td>6.57a</td>
</tr>
<tr>
<td>Gh</td>
<td>4.94cd</td>
<td>5.63a</td>
<td>5.37a</td>
<td>5.33ab</td>
<td>7.32b</td>
<td>4.43b</td>
</tr>
<tr>
<td>Fl</td>
<td>4.55cd</td>
<td>6.14a</td>
<td>6.36a</td>
<td>5.91a</td>
<td>6.20bc</td>
<td>6.08a</td>
</tr>
<tr>
<td>Fm</td>
<td>5.28c</td>
<td>6.44a</td>
<td>6.32a</td>
<td>6.10a</td>
<td>5.46c</td>
<td>6.00a</td>
</tr>
<tr>
<td>Fh</td>
<td>7.22a</td>
<td>6.45a</td>
<td>6.85a</td>
<td>4.75ab</td>
<td>5.80c</td>
<td>6.57a</td>
</tr>
</tbody>
</table>

Means within the column followed by a different superscript were different significantly.

¹P < 0.01
²P < 0.5716
³P < 0.2973
⁴P < 0.0929
⁵P < 0.001
⁶P < 0.001
⁷Six months ripened untreated cheese.
There were no significant differences in flavour and aroma characteristics between the experimental cheeses and the control cheese. This was expected because of the accelerated ripening of the trial cheeses. It could have also been due to the fact that the enzyme used in this study, Flavourzyme, is a flavour enhancing enzyme and must have produced a similar flavour note in the experimental cheeses. The strongest flavour was noted in cheeses treated with κ-carrageenan capsules. This was due to the increased level of amino acid produced in those cheeses as revealed in this study (Section 4.4.2). Free amino acids are assumed to be flavour precursors in ripening cheese (Law and Wigmore, 1982). Not all panellists appreciated the stronger flavour in κ-carrageenan capsules treated cheeses. Some panellist regarded it as atypical because they were probably used to the vintage flavours of cheese.

Cheeses treated with Fm, Fl, Gh and Gm capsules scored higher for texture but not significantly different (P < 0.05) from the control cheese. The higher score for texture by these trial cheeses could be explained by the hypothesis put forward by Creamer and Olson (1982) regarding ripening cheese. According to their model, increased peptidolysis as experienced in the experimental cheeses in this study, exposed more ionic groups which would bind the available water resulting in less solvation of the remaining casein. In addition, when the elasticity of the cheese has been reduced by proteolytic cleavage of the α_{s1}-casein (mainly caused by rennet), which is regarded as a link in the protein network (McSweeney et al., 1993a), the cheese might be perceived as harder, more brittle and less elastic. This hypothesis was true for cheeses treated with Fm, Fl, Gh and Gm capsules because they were perceived as more firmer than the control cheese. The hypothesis was however not true for κ-carrageenan capsules treated cheeses probably because of the very high level of proteolysis experienced.

Overall, the difference in texture was not significant (P < 0.05) between the trial cheeses and control cheese which implies that although hydrocolloids (Sanderson and Clark, 1983) and fat (Stamponi and Noble, 1991) are known to modify food texture, the levels at which they were used in this study had little or no influence on cheese curd texture.
Except for cheeses treated with Gl capsules, all trial cheeses scored higher than control cheese for bitterness but only cheeses treated with κ-carrageenan and Gh capsules were significantly (P < 0.05) more bitter than control cheese. This could be attributable to the relatively high proteolytic activities experienced in cheeses treated with κ-carrageenan and Gh capsules leading to bitter flavour. This is consistent with earlier reports from studies with slurries and whole cheese (Kosikowski and Iwasaki, 1975; Sood and Kosikowski, 1979) where excessive proteolysis brought about bitter taste in cheese. The bitter defect is due to the production of peptides which characteristically contain a high proportion of aromatic and bulky hydrophobic amino acid residues (Richardson and Creamer, 1973). Bitterness was however least expected in this study as Flavourzyme was supposed to bring about increased proteolysis without generating bitterness according to its manufacturer, Novo-Nordisk. The bitterness experienced in cheeses treated with KI and Gh capsules therefore means that beyond a certain level of proteolysis, irrespective of the type of enzymes involved, there will always be bitterness in cheese.

There were no significant differences in overall preferences for the experimental cheeses and the control cheese except for cheeses treated with Gh and κ-carrageenan capsules. Cheeses treated with Gl capsules was the most acceptable even more than the control cheese. This is consistent with earlier studies where it was reported that gellan gum treated cheeses have superior flavour than those treated by carrageenan (Kanombirira and Kailasapathy, 1995). This is because gellan gum has excellent flavour release properties (Sanderson and Clark, 1983). Mean scores for appearance, flavour, aroma, texture (when biting) and bitter aftertaste correspond positively with the high overall acceptability of Gl capsules treated cheeses, indicating that these were strong contributors to cheese preference. Cheeses treated with Gh and κ-carrageenan capsules showed the lowest acceptability. This was definitely due to both their high levels of bitterness and poor textural appearances as observed in this study.
4.5 CONCLUSION

The results from this experiment showed that there was increased rate of proteolysis in the trial cheeses as compared to the control cheese. κ-Carrageenan produced the most unstable capsules in cheese. Gellan gum capsules and milk fat capsules showed very similar behaviour but both were less unstable than κ-carrageenan in cheese. Cheeses treated with κ-carrageenan capsules exhibited the highest rate of casein breakdown and proteolysis. The disruption of κ-carrageenan and gellan capsules in cheese curd was related to the presence of lactic acid and for gellan capsules also influenced by the presence of other ions such as protons in the curd. Degradation of casein by κ-carrageenan encapsulated enzymes was apparently influenced by the κ-carrageenan-casein interaction.

Determination using Texture Profile Analyser (TPA) indicated that application of gum or milk fat enzyme capsule to cheese did influence their textural qualities significantly (P<0.05). The change in cheese texture was however not significant (P<0.05) according to the sensory evaluation results. This is an indication that objective tests can pick up certain differences that are difficult to detect through subjective methods. The variations noted in the sensory quality of the ripened cheeses were basically a result of the activity of the enzyme applied rather than the gums or milk fat. Cheeses treated with κ-carrageenan capsules had the lowest mean score for most sensory attributes due to the very high rate of proteolysis experienced.

This study has shown that incorporation of enzyme encapsulated in gellan, milk fat and κ-carrageenan in cheese during manufacture can lead to acceleration of ripening because of the increased proteolysis. κ-Carrageenan showed more potential as an enzyme encapsulant because they brought about the highest rate of proteolysis. Unfortunately, the increased proteolysis was accompanied by deterioration in texture and production of bitter taste in cheese. This is a set back with this technique of accelerating cheese ripening which requires further investigation before the technique can be adopted for industrial applications.
OVERALL SUMMARY

Food gum gels were assessed for their stabilities when subjected to conditions similar to those encountered during cheese making and ripening. Gels were made from κ-carrageenan, gellan, agar, ι-carrageenan, alginate and κ-carrageenan-locust bean gum. The gels were treated in solutions of different acidities (0.4, 0.8, 1.2%) and varied pHs (6.2, 5.5, 4.8). After 24 and 48 h, the gels were tested by a Texture Profile Analyser (TPA) to determine their strengths. Gels from all tested gums showed reduced strength upon treatment as compared to the control gels. The strength of most gum gels was unaffected by varying either the acidities or pHs of the solution in which they were treated. Only κ-carrageenan gels significantly changed strength when both acidities and pHs of the treatment solutions were changed.

In the next experiment, the ability of gum gels to encapsulate and maintain the viability of enzymes was assessed. Enzyme, Flavourzyme, was entrapped in κ- and ι-carrageenan, gellan, alginate, agar and ι-carrageenan-locust bean gum gels and made into capsules of diameter 348-524 μm. The specific enzyme activity of the encapsulated enzymes was also measured. Enzyme encapsulated in κ-carrageenan, gellan and alginate gels showed the highest specific activity, 20.00, 7.69, and 7.14 ΔA/mgN respectively. Agar gel entrapped enzyme showed the least specific activity (1.00 ΔA/mgN).

Gellan and κ-carrageenan gums were selected based on the findings of the preceding experiments for further trials in cheese manufacture. Cheeses were manufactured from cheese milk to which was incorporated enzyme capsules. Retention of the added enzyme capsules in cheese curd and loss of enzyme in cheese whey were determined. Capsules retention in cheese curd for gellan, κ-carrageenan and milk fat were 91.5, 90.0 and 73.5% respectively. Enzyme losses in cheese whey was 8.86, 5.62 and 17.93 for κ-carrageenan, gellan and milk fat respectively.

Finally, the effect of adding encapsulated enzyme on cheese ripening was assessed. Proteolysis in ripening cheeses was measured. Breakdown of casein was monitored by
the HPCE method. Release of free amino acid during cheese ripening was determined by reacting them with TNBS. All cheeses treated with encapsulated enzyme had higher rates of proteolysis than the untreated cheese almost throughout the ripening period. Rate of proteolysis was highest in κ-carrageenan capsules treated cheeses. The addition of enzyme capsules to cheese had no significant effect on the microbial growth in cheese. Similarly, cheese texture and sensory quality were not influenced by the addition of either gums or milk fat capsules. Differences in textural and sensory quality between trial and control cheeses were consistent with release of enzymes from capsules.
OVERALL CONCLUSIONS

Gels of κ-carrageenan, agar, alginate, λ-carrageenan and κ-carrageenan-locust bean gums showed reduction in strength when treated in acidified solutions (0.4, 0.8, 1.2% acidity) and solutions with adjusted pH (6.2, 5.5, 4.8) as compared to control gels (treated at pH 7.0 or 0.0 % lactic acid). Gellan gum gel showed an increase in strength upon similar treatments. Varying acidity (0.4 - 0.8 -1.2%) and pH (6.2 -5.5 - 4.8) of the solutions did not considerably affect the strength of most of the gum gels tested except for κ-carrageenan. This indicated that κ-carrageenan could possibly break down under conditions similar to those encountered during Cheddar cheese manufacture. Thus, κ-carrageenan was considered suitable for enzyme encapsulation for incorporation into cheese to accelerate ripening.

Further testing of the gums indicated that enzyme encapsulated in gellan and κ-carrageenan gel capsules had higher activity than for the other gums tested. Therefore, the use of these two gums for encapsulating enzymes could possibly be economical in terms of reduced amount of enzyme required for encapsulation.

The levels of retention of gum capsules in cheese curd were very good. κ-Carrageenan and gellan capsules had higher retention, 90.0 and 91.2 % respectively, than milk fat capsules (73.5%). Enzyme losses from gum gel capsules were also lower, 5.62 and 8.66 % for gellan and κ-carrageenan gums respectively, than for milk fat capsules (17.93%). These findings indicate that use of gum gels as enzyme encapsulant for cheese application posses very low risk of enzyme contamination of cheese whey hence is suitable for cheese application.

Studies reported in this thesis revealed that the encapsulation of enzyme in food gum gels for the purposes of speeding up the processes responsible for cheese ripening is feasible. Cheeses treated with enzyme capsules showed increased rate of proteolysis as compared to the untreated cheese. This study also revealed that a successful application of gums for encapsulating enzymes meant for use in accelerating cheese ripening is influenced greatly by the nature of the gum itself.
Cheeses treated with κ-carrageenan showed the highest rate of proteolysis as compared to those treated with gellan and milk fat capsules. Conditions in cheese such as the presence of ions and lactic acid appeared to have influenced the stability of gum capsules in cheese. Interaction between milk protein and gum capsules most likely enhanced the interaction between the encapsulated enzyme and milk proteins. In other words, interaction between gum and milk proteins appeared to have facilitated the increased proteolysis by κ-carrageenan.

Gum gels which get disrupted under the cheese manufacturing conditions appeared to be more suitable for encapsulating enzyme to be used for accelerating cheese ripening. However, the use of capsule from a gum which is easily disrupted under cheese manufacturing conditions may lead to fast release of enzyme and cause excessive proteolysis early during ripening. This is similar to what was seen in this study for κ-carrageenan. Cheeses that experienced high levels of proteolysis scored poorly for texture and sensory properties. This was one major drawback observed from this study. It is therefore important in future studies to consider combining such unstable gums with gums that produce more stable gels. For instance capsules made by mixing κ-carrageenan and gellan gums.
RECOMMENDATIONS FOR FURTHER RESEARCH

Acceleration of cheese ripening by use of enzymes encapsulated in gums provides a very new avenue to this rather old concept of maturing cheese quickly. Use of liposomes and milk fat for encapsulating enzymes has been considerably researched but the mechanism of enzyme release from liposomes has not yet been fully elucidated. The lack of proper knowledge on liposomes release mechanisms has been one of the obstacles to any further interest in this technique. Similarly, for gums to receive any attention especially from cheese producers, more understanding is required about the molecular behaviour of different gums in cheese and the mechanism of their capsules breakdown. The view that lactate and pH change play major roles in gum capsules breakdown in cheese curd may not be sufficient. There could be other mechanisms as well.

Enhanced ripening of cheese has not only resulted in production of piquant flavour quickly but has also often led to an unacceptable change in cheese texture and sensory characteristics. This is similar to what was observed in cheeses treated with κ-carrageenan enzyme capsules in this study. This normally results from excessive enzyme activity caused by a one-step kind of capsule breakdown. Proper development of desirable flavour during accelerated ripening requires a slow but sustained release of the encapsulated enzyme. Because one gum can modify the property of another gum, the possibility of combining a relatively stable gum with a very unstable gum should be investigated. This could reduce the negative effects of accelerated ripening on cheese textural and sensory properties.

Due to the relatively high cost of suitable enzyme for accelerating cheese ripening, the cost of enzyme encapsulation in gum gels requires investigation vis-à-vis the cost of ripening cheese by the conventional way. This would throw some light on the actual economic gain that may be obtained by employing this technique rather than ripening in the conventional method.
PLEASE NOTE

The greatest amount of care has been taken while scanning the following pages. The best possible results have been obtained.
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NOVO Nordisk A/S: Preliminary Product Information, Bagsvaerd, Denmark.


PLEASE NOTE

The greatest amount of care has been taken while scanning the following pages. The best possible results have been obtained.
Flavourzyme™

Application
Flavourzyme is a fungal protease/peptidase complex developed for hydrolysis of proteins under neutral or slightly acidic conditions. Flavourzyme can be used for debittering of bitter protein hydrolysates at low degrees of hydrolysis and for extensive hydrolysis of proteins resulting in taste development.

Characteristics
Flavourzyme is produced by fermentation of a selected strain of *Aspergillus oryzae* and contains both endoprotease and exopeptidase activities.

The optimal pH for the enzyme complex is in the range of 5.0-7.0. The optimal pH for the exopeptidase is approx. 7.0, as determined by application trials. The optimal pH for debittering is also approx. 7.0.

The optimal temperature for the enzyme complex as well as for the exopeptidase is around 50°C.

Dosage
For debittering, Flavourzyme can be used at dosages of 5-20 LAPU/g protein. For extensive hydrolysis, dosages of 20-50 LAPU/g protein are recommended. The optimal dosages must be determined in each individual case. For further information on the use of Flavourzyme, please see the leaflet "Extensive Hydrolysis of Proteins with Flavourzyme" (B 829), which is available on request.

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![Graphs showing enzyme activity and temperature effects](image)

**Fig. 1.** Influence of pH on the activity of Flavourzyme.
- Substrate: 8% soy protein isolate
- Enzyme conc.: 33 LAPU/g protein
- Temperature: 50°C
- Method: TNBS

**Fig. 2.** Influence of temperature on the activity of Flavourzyme.
- Substrate: 8% soy protein isolate
- Enzyme conc.: 33 LAPU/g protein
- pH: 7.0
- Method: TNBS

**Fig. 3.** Hydrolysis of various proteins with Flavourzyme.
- Substrate conc.: 8% protein
- Enzyme conc.: 37 LAPU/g protein
- Initial pH: 7.0 (no adjustment of pH during hydrolysis)
- Temperature: 50°C
- Method: TNBS

---

*Note: Novo Nordisk does not guarantee that the products can be used as described above without proper supervision.*
**Inactivation**
Flavourzyme can be inactivated at 85°C for 5 minutes.

**Description**
Flavourzyme MG is a brown, free-flowing, non-dusting microgranulate, produced in two types:

- type A: ................ granulated on NaCl
- type B: ............... granulated on wheat grits

**Specification**
The activity of Flavourzyme MG is specified for each individual batch and is expressed in LAPU/g. One LAPU (Leucine Aminopeptidase Unit) is the amount of enzyme which hydrolyzes 1 μmole of L-leucine- p-nitroanilide per minute in Novo Nordisk's analytical method AF 298/1, which is available on request.

The product complies with FAO/WHO JECFA and FCC recommendations for food grade enzymes, supplemented with maximum limits of $10^4$/g for total viable count and $10^3$/g for moulds. The product is GRAS.

**Handling**
The product is non-flammable and safe when used according to directions. Proteolytic enzymes may irritate skin and eyes and enzyme dust or enzyme-containing aerosol may cause sensitization when inhaled.

Observe standard handling precautions to avoid direct contact with the product or inhalation of dust from the dried product. In case of accidental spillage or contact with the skin or eyes, rinse promptly with water.

A separate leaflet, «How to handle powder/granulated Novo Nordisk enzymes safely» (B 143), is available on request.

**Storage**
The product should be kept dry and not too warm.

**Packing**
Flavourzyme MG is available in 40-kg fibre drums.

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**Enzyme Business**
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Denmark
Tel. +45 4444 8888
Fax +45 4444 1021
Telex 37560

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Sample Rate: 200.00 pps  Test Time: 21.50 s
Force Threshold: 20.0 g  Dist. Threshold: 0.50 mm
Sample Area: 1.00 mm²  Contact Force: 5.0 g

T.P.A
SPEED: 1.0 mm/s  PRE TEST SPEED: 5.0 mm/s  POST TEST SPEED: 5.0 mm/s
TRIGGER TYPE: Auto @ 5 g  DISTANCE: 5.0 mm
TIME: 5.00 s


---

Springiness: 0.737  Gumminess: 262.305  Fracturability: N/A  N/A
Cohesiveness: 10.437  Adhesiveness: -3.250  Hardness: 601.2 g  N/A
Chewiness: 193.775  Initial Modulus: N/A

---

Areas (g s)
1. 2186.211
2. 956.030
3. -3.250

Peaks (g)
1. 501.2
2. 470.4
3. N/A

Strain:
N/A

Initial Stress:
1.142E+007 dyns/cm²

Product Height:
N/A

Compression:
5.000 mm

Resilience:
0.282
QUESTIONNAIRE FOR CHEESE TASTING

Name ______________________ Date ___________

Please evaluate the flavour and texture of these cheese samples. Make a vertical line on the horizontal line to indicate your rating of the flavour, aroma and texture of each sample. Label each vertical line with the code number of the sample it represents.

Appearance
(texture):   crumbly       not crumbly

Flavour:   slight       intense

Aroma:   slight       intense

Texture
(when biting):

soft       hard

Bitter aftertaste:

slight       intense

Overall Acceptance:

very unacceptable       very acceptable

THANK YOU FOR YOUR TIME.
APPENDIX 4

Analysis Data sheet Printout from HPCE for a sample

Date File C:\HCHEM\DATA\CHAK\WEL0.D Sample Name: casein
Instrument 1 15/11/96 11:30:14 AM lapigo

Injection Date : 15/11/96 10:55:53 AM  Seq. Line : -
Sample Name : casein unused Vial : 7
Acq. Operator : lapigo Inj : -
Acq. Method : A:\HENRYCAS.M
Last changed : 15/11/96 10:52:51 AM by lapigo
(modified after loading)
Analysis Method : A:\HENRYCAS.M
Last changed : 15/11/96 11:30:08 AM by lapigo
(modified after loading)

Sorted by Signal
Area Calculation Mode : Measured Area
Calib. Data Modified : Friday, 15 November, 1996 11:30:07 AM
Multiplier : 1.000000
Dilution : 1.000000
Uncalibrated Peaks : not reported

Signal 1: DAD1 B, Sig=214.2 Ref=off

<table>
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<th>MT</th>
<th>Type</th>
<th>Area [mm²]</th>
<th>Amt/Area [ug/ml]</th>
<th>Amount Grp</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.145</td>
<td>MM</td>
<td>293.64941</td>
<td>2.30516</td>
<td>676.91034</td>
<td>b-casein</td>
</tr>
</tbody>
</table>

Totals :
676.91034

*** End of Report ***
Appendix 5a. Casein Standards
Appendix 5b (i).

Appendix 5b (ii).

Appendix 5b (iii).
Appendix 5c (i).

Appendix 5c (ii).

Appendix 5c (iii).
Appendix 5d (i).

Appendix 5d (ii).

Appendix 5d (iii).
Appendix 5f (i)

Appendix 5f (ii)

Appendix 5f (iii)
Gellan High (Gh)

Appendix 5g (i)

Appendix 5g (ii)

Appendix 5g (iii)
Milkfat Low (MI)

Appendix 5h (i)

Appendix 5h (ii)

Appendix 5h (iii)
Milkfat Medium (Mm)

Appendix 5i (i)

Appendix 5i (ii)

Appendix 5i (iii)
Control Cheese (CC)

Appendix 5k (i).

Appendix 5k (ii).

Appendix 5k (iii).
Appendix 5j (i)

Appendix 5j (ii)

Appendix 5j (iii)