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RENNET COAGULATION PROPERTIES OF HEATED MILKS

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF TECHNOLOGY IN FOOD TECHNOLOGY

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1995

ABSTRACT

The effects of heat treatment at temperatures in the range 80 - 140°C for 4 s in a spiral flow indirect UHT plant on (i) the denaturation of whey proteins, (ii) their association with the casein micelles and incorporation into rennet gels and (iii) rennet coagulation properties of skim milk were determined.

The extent of denaturation of β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), as determined by the decrease in the amounts of native protein (Native-PAGE) in the ultracentrifugal supernatants (100,000*g* for 1 h) of heated milks, increased with the severity of heat treatment. At all temperatures, β-Lg was more sensitive to thermal denaturation than α-La. The extent of association of β-Lg and α-La with the casein micelles, as determined by the total amounts of these proteins (SDS-PAGE) remaining in the ultracentrifugal supernatants of heated milks, also increased with the severity of heat treatment. At any given temperature, association of these proteins with casein micelles was much less than the amount that denatured. The extent of incorporation of β-Lg and α-La into rennet gels increased with temperature and could be related to the levels of denaturation of β-Lg and its association with casein micelles.

Heat treatment impaired the rennet coagulation properties of milk as indicated by an increase in gelation times and a decrease in gel strengths (both determined using the Bohlin VOR Rheometer). There was a close correlation between the extents of association of β-Lg with the casein micelles and the changes in gelation time or gel strength. When heated milks were acidified to pH 5.5 and re-neutralised to pH 6.5 (pH cycled), the adverse effects of heat treatment on rennet coagulation were reduced, except for those milks heated at temperatures above 120°C. The rennet coagulation properties of heated milks were markedly improved by addition of low concentrations of $CaCl_2$, but no additional improvement

resulted when $CaCl_2$ addition was combined with pH cycling.

Concentration of skim milk by ultrafiltration (UF) lengthened gelation time but increased gel strength, the effect being dependent on the volume concentration ratio (VCR). Heat treatment of milk (140°C for 4 s) before or after UF increased gelation times and lowered gel strengths with weaker gels being formed from milks heated prior to UF. pH cycling of heated milk before UF or of heated UF concentrates had an adverse effect on rennet coagulation properties of the UF concentrates. When 3X UF concentrate was heated at temperatures in the range 80 - 140°C for 4 s, gelation time did not change with temperature between 80 and 120°C but more severe heat treatments caused an increase. In contrast, the gel strength decreased gradually with increase in heating temperature. These changes in rennet coagulation properties were related to the extent of denaturation of β-Lg and its association with the casein micelles.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my supervisor, Dr. Harjinder Singh, for introducing me to this subject and for his unfailing commitment, support and expert advice in all stages of this research project. I am also grateful for the encouragement and constructive criticism received from my co-supervisor, Mr Rod Bennett, especially during compilation of this manuscript.

Ms June Latham is greatly acknowledged for her help on the Bohlin Rheometer and her patience and willingness to be called up at home (often at odd hours) to come and tend to minor difficulties with the machine. The technical assistance received from Messrs Steve Glasgow, Alistair Young, Garry Radford and Byron McKillop is greatly appreciated as is the help received from other members of staff in the Food Technology department.

I would like to thank my fellow graduate students for their friendship and helpful discussions on aspects of my study particularly David Oldfield for his assistance in the techniques of gel electrophoresis.

I enjoyed some great Kiwi hospitality over my two years in New Zealand from the following families to whom I extend a big thank you: Mr and Mrs W. Gartner, Mr and Mrs R. van Laar-Veth, Mr and Mrs A. Schaw and Mr and Mrs J. van Laar.

Special thanks are due to Jackie Gezimati for her continued friendship and to my family for their support and encouragement throughout my studies.

Finally, I would like to thank the New Zealand Ministry of Foreign Affairs and Trade for financing both my stay and study in New Zealand through the award of an ODA Scholarship.

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CHAPTER 1

INTRODUCTION

The yield of dairy products has assumed increasing importance as economic factors have reduced the potential profit returns (Banks and Muir, 1985). As a result, widespread attempts are being made to increase processing efficiency and to evaluate new technology. In cheesemaking, much research attention is focused on trying to improve yields through incorporation of whey proteins into cheese. Incorporation of these proteins, which account for approximately 20% of the total protein content of bovine milk, would result in an increase in the final yield of cheese in the order of 12% (Banks *et al.* 1987) thus greatly improving the efficiency of cheese production while also improving the products' nutritive value.

One way to incorporate whey proteins into cheese is to heat treat the milk which denatures the whey proteins, subsequently resulting in their incorporation into the coagulum. In addition, high heat treatment of milk would also give a low bacterial load with an extended storage life permitting milk to be transported long distances or held for long periods before cheese manufacture. However, it is well established that heat treatment of milk adversely affects the rennet coagulation times and the properties of the curd; heated milks have longer coagulation times and form weaker curds than the unheated milk. The effect of heating on renneting is believed to arise mainly from the formation of complexes between denatured whey proteins and casein micelles although changes in calcium phosphate equilibrium are also involved. However, the exact mechanisms by which these changes alter the renneting reaction are not fully understood and are currently the subject of ongoing research.

Ultrafiltration (UF) provides another possible method for increasing yields in cheese manufacture. Major problems generally encountered in the

manufacture of hard cheeses from UF milk include short coagulation times and high curd firmness which makes cutting of the curd in conventional cheese production equipment difficult.

Since UF and heat treatment have opposite effects on the rennet coagulation of milk, it is likely that heat treatment of UF milk may prove effective in correcting the curd firmness problem of curds made from UF milk. Little information is available on the effects of heat treatment of UF milk on its rennet coagulation properties.

A better understanding of the influence of heat treatment of normal milk and UF milk on the rennet coagulation properties should provide useful information on ways of utilising heated milks for manufacture of cheese containing denatured whey proteins and may enable the production of a range of new products, e.g. milk protein concentrate powders.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Milk

Milk is a complex system of protein aggregates, soluble proteins, salts, lactose, fat and water. It is a very versatile raw material used for the manufacture of a wide variety of products due to the ability of its components to withstand various process manipulations. Cheese is one of the leading dairy products and its manufacture is dependent on the milk proteins (especially the caseins) and to a lesser extent the milk salts.

2.2 Milk proteins

Bovine milk contains on average about 3.3% protein. About 80% of the milk protein consists of caseins, a group of phosphate-containing milk specific proteins that precipitate upon acidification to pH 4.6. Almost all casein in milk is present in casein micelles. Four kinds of polypeptide chains, designated α_{s1}-, α_{s2}-, β-, and κ-caseins, together with some derivatives formed by proteolysis of these chains (e.g. γ-caseins) have been identified. The proteins remaining in solution at pH 4.6 are called whey proteins and consist of a diverse group, including β-lactoglobulin (β-Lg), bovine serum albumin (BSA), α-lactalbumin (α-La), immunoglobulins and small molecular weight peptides, derived by proteolysis of some of the caseins (Walstra and Jenness, 1984).

2.2.1 Casein micelles

Approximately 95% of the casein exists as coarse colloidal particles in normal milk. These colloidal particles, (casein micelles) range in diameter from 50 - 300nm (Schmidt *et al.* 1973). The micelles consist of ~92% protein (casein) and 8% inorganic salts, mainly calcium phosphate (Whitney, 1988).

The structure of the casein micelles

The structure and behaviour of casein micelles is a topic of on going research and insight into their behaviour is very important to the advancement of dairy technology.

The exact structure of the casein micelle has not been fully resolved with several models having been proposed in the past three decades (Rose, 1969; Waugh *et al.* 1971; Slattery and Evard, 1973; Schmidt, 1980; Walstra and Jenness, 1984).

Although there is some debate, the casein micelle is generally viewed as being composed of smaller units called sub-micelles. There is no clear agreement on their size; diameters of 10 - 20nm and molecular weights of 250,000 - 2000,000 have been reported. Sub-micelles, like globular proteins, have a dense hydrophobic core, in which are tucked most of the hydrophobic parts of the casein molecule and a less dense, hydrophillic outer layer, containing most of the acidic (carboxylic and phosphoric) and some of the basic groups. Each sub-micelle contains different casein molecules and is unique in composition. Generally, the principal caseins are in a constant ratio in a sub-micelle although variations are common, particularly with κ-casein.

Individual sub-micelles are believed to be linked together by colloidal calcium phosphate (CCP) to form the casein micelles. The nature of the bonds that enable CCP to link adjacent sub-micelles are not known although hydrophobic and hydrogen bonds are also thought to contribute to micellar stability.

In the presence of calcium and phosphate, aggregation of sub-micelles would continue to the extent of gel formation were it not for the presence of κ-casein on the surface of the micelle (McGann *et al.* 1980; Rollema *et al.* 1981; Donnelly *et al.* 1984) which acts as a 'protective colloid'. The

stability effect arises because κ-casein, as shown by its peptide sequence (Mercier *et al.* 1973), is divided into two distinct regions, namely the hydrophobic para-κ-casein (residues 1 - 105) and the hydrophillic macropeptide or glycopeptide (CMP or GMP, residues 106 - 169). In its natural position on the surface of the micelles, κ-casein is linked to the remainder of the micelle via the para-κ-casein part of the molecule, so that the macropeptide protrudes into the surrounding solution. This hydrophillic moiety interacts with the solvent to stabilise the micelles (Walstra, 1979; Walstra *et al.* 1981; Horne, 1984, 1986; Holt and Dalgleish, 1986; Dalgleish and Holt, 1988).

The structure shown in Figure 2.1 was suggested by Walstra and Jenness (1984) and was mainly derived from the models of Schmidt and Payens (1976), Slattery (1976) and Walstra (1979). Walstra and Jenness (1984) consider this model in best agreement with the numerous observations on properties and stability of the casein micelles, including renneting. Most aspects of this model are almost common ground among researchers.

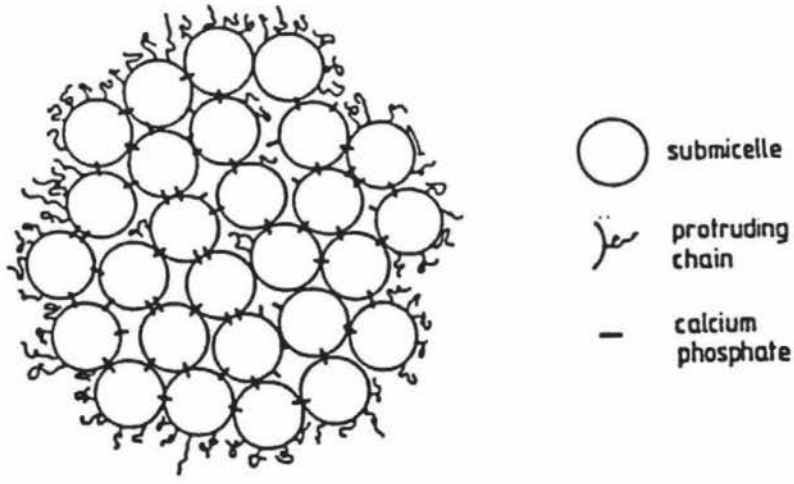

Figure 2.1: Structure of the casein micelle (from Walstra and Jenness, 1984).

2.2.2 Whey proteins

Traditionally, the term "whey proteins" has been used to describe those milk proteins remaining in the whey after precipitation of the caseins at pH 4.6 (20°C). Whey proteins may be subdivided into different groups which include β-lactoglobulin (β-Lg), α-lactalbumin (α-La), serum albumin (BSA) and immunoglobulins. The proteose-peptones are now assigned to the β-casein family as they are products of β-casein degradation (Walstra and Jenness, 1984).

β-Lactoglobulin (β-Lg)

β-Lg accounts for about 50% of the total whey protein in bovine milk. Seven genetic variants A, B, C, D, E, F, and G of bovine β-Lg have been identified. β-Lg has a monomeric molecular weight of 18, 000 Da, but exists as a stable dimer between pH 5.5 and 7.5. β-Lg contains five cysteine residues per mole, of which four are involved in disulphide linkages. It has a single free thiol group which is of great importance for changes occurring in milk during heating since it is involved in reactions with other proteins, notably κ-casein and α-La (Walstra and Jenness, 1984). The physico-chemical properties of β-Lg have been extensively reviewed (e.g. Tilley, 1960; Townend *et al.* 1969; McKenzie, 1971; Green *et al.* 1979).

α-Lactalbumin (α-La)

α-La is the second most abundant of the whey proteins accounting for 20% of them. Like the other whey proteins, α-La is a globular protein and it has a molecular weight of 14,000. There are four interchain disulphide bonds, but no sulphydryl groups are present in α-La which does not polymerise upon heat treatment.

Bovine serum albumin (BSA)

BSA is identical to the serum albumin found in the blood and has a molecular weight of 66, 267 (Eigel *et al.* 1984). It is synthesised in the

liver and gains entrance to milk through the secretory cells (Walstra and Jenness, 1984) and accounts for about 5% of the total whey proteins. BSA has one free thiol (Cys 34) and 17 disulphide linkages, which hold the protein in a multiloop structure.

Immunoglobulins

Immunoglobulins are antibodies synthesised in response to stimulation by macromolecular antigens foreign to the animal. They account for up to 10% of the whey proteins and are polymers of two kinds of polypeptide chains, light (L) of MW 22,400 and heavy (H) of MW 50 - 60,000 Da. The light and heavy chains (two of each) are joined by disulphide linkages to form the basic immunoglobulin structure. Four types of immunoglobulins have been found in bovine milk, IgM, IgA, IgE, and IgG (Eigel *et al.* 1984).

Other Whey Proteins

Several other whey proteins are found in small quantities in whey and these include two iron binding proteins, lactoferrin and transferrin, and a group of acyl glycoproteins.

2.3 Milk Salts

Milk salts consist mainly of chlorides, phosphates, citrates and bicarbonates of sodium, potassium, calcium, and magnesium (Pyne, 1962). Their practical importance arises largely from their marked influence on the condition and stability of the milk proteins, particularly caseins.

Some of the milk salts (e.g. the chlorides, sulphates and compounds of sodium and potassium) are soluble and are present almost entirely as ions dissolved in milk whey. Others, calcium and phosphate in particular, are much less soluble and at the normal pH of milk exist partly in dissolved and partly in insoluble (i.e. colloidal) form in close association with the caseins. The partition of calcium phosphate between the whey and milk colloids is very important since it significantly influences the properties of

milk depending on the phase in which it is situated.

2.4 Effect of heat on milk

A number of changes occur in milk systems during heating; these changes have been extensively reviewed (e.g. Fox, 1981; Singh, 1988; Singh and Fox 1989; Singh *et al.* 1989; Singh and Creamer, 1992).

Significant changes occurring above 60°C include whey protein denaturation, interaction between denatured whey proteins and the casein micelle, and the conversion of soluble calcium, magnesium, phosphates, and citrates to the colloidal state.

2.4.1 Changes in milk proteins

Caseins

Generally, casein micelles are very stable at high temperatures due to their lack of secondary or tertiary structure and the presence of a rather complex quaternary structure. As such they can withstand commercial sterilisation and UHT heat treatments although some changes (mainly hydrolytic) such as dephosphorylation and proteolysis as well as changes in micellar structure (zeta potential, hydration changes and association-dissociation reactions) do occur at these severe heating temperatures (Fox, 1981; Singh, 1988; Singh and Creamer, 1992). The most pronounced effect of high temperatures on casein micelles is increase in size (Wilson and Herreid, 1961; Hostettler *et al.* 1965; Josephson *et al.* 1967; Carroll *et al.* 1971; Creamer and Matheson, 1980) which is probably due to deposition of denatured whey proteins on the micellar surfaces and precipitation of calcium phosphate.

Whey proteins

The heating of milk at temperatures above 60°C causes denaturation of the heat labile whey proteins. Protein denaturation has been defined by some

authors (de Wit, 1981; Mulvihill and Donovan, 1987) as the unfolding of the native globular form into a less ordered structure. The unfolded proteins then proceed by an entirely separate step to an aggregated form. Once denatured, the whey proteins may bind to the casein micelles (Smits and van Brouwershaven, 1980; Singh and Fox, 1987ab) or simply associate with themselves to form polymeric products.

Thermal denaturation of whey proteins has been extensively researched and has been reviewed by Mulvihill and Donovan (1987). The order of sensitivity of the various whey proteins to heat has been reported to be immunoglobulins > BSA > β-Lg (variant A>B) > α-La, as determined using protein precipitation methods (Larson and Rolleri, 1955; Dannenburg and Kessler, 1988). However, α-La has been shown to be the least stable protein using differential scanning calorimetry (DSC) (Ruegg *et al.* 1977; Bernal and Jelen, 1985).

pH, ionic strength and the concentrations of calcium and protein markedly influence the extent of denaturation of the whey proteins (Hillier *et al.* 1979; Park and Lund, 1984; Dannenburg and Kessler, 1988). The rates of denaturation of both β-Lg and α-La are higher at pH 6.5 than at pH 4.5; however, denaturation of both the proteins is much slower at pH 2.5 (Harwalkar, 1986). Increasing the calcium concentration up to 4mg\ml tends to retard the denaturation of both proteins, but further increase has little effect. Increasing the protein concentration decreases the rate of denaturation of β-Lg, but increases the denaturation of α-La (Hillier *et al.* 1979). Heat denaturation of whey proteins is also influenced by lactose and other sugars, polyhydric alcohols and protein modifying agents (Hillier *et al.* 1979; Bernal and Jelen, 1985; Donovan and Mulvihill, 1987).

The temperature of denaturation for α-La is lower (68°C) than that for β-Lg (74°C) but extensive renaturation (80 - 90%) of α-La can occur after cooling (Ruegg *et al.* 1977; de Vit *et al.* 1985). This renaturation may

account for the observation that α-La is the most resistant of the whey proteins to denaturation by heat.

Denaturation of whey proteins may be assessed through measurement of loss of solubility, reactivity of thiol groups, electrophoretic analysis, loss of antigenic activity and differential scanning colorimetry (DSC). These methods are based on different physical or chemical properties of the protein, and so it is difficult to decide whether these measurements of denaturation are comparable under different conditions.

Casein / whey protein interactions

Heating of milk above 90°C results in whey protein denaturation and association with κ-casein on the surface of the casein micelles, giving the appearance under an electron microscope of threadlike appendages, protruding from the micelles (Creamer and Matheson, 1980; Mahammad and Fox, 1987). The type of association between whey proteins and caseins depends on the severity of the heat treatment (Hostettler *et al.* 1965; Josephson *et al.* 1967) and involves both disulphide and hydrophobic interactions (Smits and van Brouwershaven, 1980; Singh and Fox, 1987b).

Not all the denatured whey proteins complex with the casein micelles. Some remain in the serum where they may form aggregates with other whey proteins or with serum κ-casein. The extent of association of denatured whey protein with casein micelles is markedly dependent on the pH of the milk prior to heating. Heating at pH values less than 6.7 results in a greater quantity of denatured whey proteins associating with casein micelles, whereas, at higher pH values, whey protein\κ-casein complexes dissociate from the micelle surface, apparently due to dissociation of κ-casein (Singh and Fox, 1985, 1986).

2.4.2 Changes in milk salts

Increase in temperature induces changes in the nature of some of the milk salts (namely those of calcium, magnesium, citrate and phosphate) and causes them to move from the soluble to the colloidal phase. As a result of its association with casein micelles, heat-precipitated calcium phosphate does not sediment (Evenhuis and de Vries, 1956). On subsequent cooling, some of the indigenous or heat precipitated calcium phosphate may redissolve, especially if the heating temperatures are less than 85°C (Kannan and Jenness, 1961). Certain other changes, however, such as loss of CO_2, and formation of very insoluble types of calcium phosphate such as hydroxyapatite (at more severe heating temperatures) are for all practical purposes irreversible. Heat treatments have little effect on the monovalent ions (Na^+, K^+, and Cl^-).

2.5. Rennet coagulation of milk

The clotting of milk by the specific action of selected proteolytic enzymes forms the basis for the manufacture of most cheese varieties. It is one of the oldest operations in food technology, having a history of some thousands of years (Dalgleish, 1982).

Coagulation is initiated by the action of proteolytic enzymes called rennets. These rennets consist of the enzymes chymosin and pepsin, and are traditionally prepared from the stomachs of calves, kids, lambs or other mammals in which rennins are the principal proteinases. Increased production of cheese worldwide, coupled with a trend to slaughter calves at an older age, has led to a search for rennet substitutes. The most successful of these substitutes over the past two decades have been bovine, porcine and chicken pepsins as well as the acid proteinases from microbial sources such as *Mucor pucillus*, *Mucor meihei* and *Endiothia parasitica*. Recently, the gene for chymosin has been cloned and expressed in microogarnisms (e.g. yeasts and *E. Coli*) to allow production of rennets containing chymosin only (Foltmann, 1987). The products of cloning are

now becoming available and used commercially with promising early results (Hicks *et al.* 1988; Bines *et al.* 1989; Pszczola, 1989). General and molecular aspects of rennets have been reviewed by Foltmann (1987).

2.5.1 Mechanism of coagulation

The rennet coagulation of milk occurs in phases; a primary enzymatic phase, a secondary non-enzymatic phase and a less clearly defined tertiary phase (Berridge, 1942; Dalgleish, 1982, 1992).

Primary phase

The primary phase of rennet coagulation of milk involves the specific hydrolysis of κ-casein in the region of the $Phenylalanine_{105}$ - $Methionine_{106}$ bond (Jolles *et al.* 1968), Phe_{105} supplying the carboxyl group and Met_{106} the amino group, producing two peptides of contrasting physical and chemical properties. The glycomacropeptide (GMP) moiety, which is comprised of amino acid residues 106 - 169 is hydrophillic, soluble in trichloroacetic acid (Armstrong *et al.* 1967), and has a molecular weight of 3000 to 6000. GMP diffuses away from the casein micelle after splitting from κ-casein and into the serum. As a result, its stabilising influence is lost, and the micelles can begin to coagulate once sufficient κ-casein has been hydrolysed (Dalgleish and Holt, 1988). The second peptide, para-κ-casein, which consists of amino acid residues 1 - 105, has a higher molecular weight and is strongly hydrophobic. Para-κ-casein remains attached to the micelles.

For milk to be satisfactorily clotted, the attacking protease must be very specific for the Phe_{105} - Met_{106} bond of κ-casein and the capacity of the proteases for more general proteolysis must be low to avoid non-specific formation of soluble peptides which will be lost in the whey and thus reduce yields during cheese manufacture. Lack of substrate specificity is one of the main reasons behind the failure of many rennet substitutes.

Casein micelles possess an overall negative charge on their surface which results in micellar repulsive forces which prevent close approach and coagulation of the micelles in the stable milk system. The bulk of this charge is carried by the GMP of κ-casein which because of its hydrophilicity interacts well with the aqueous whey to protect against any micellar aggregation. Loss of GMP during the primary phase of renneting results in loss of about half this charge (Green and Crutchfield, 1971; Green, 1973; Pearce, 1976; Darling and Dickson, 1979; Dalgleish, 1984) while the micellar surface becomes more hydrophobic due to the accumulation of para-κ-casein. Consequently, coagulation becomes possible.

Secondary phase

κ-Casein hydrolysis during the primary phase alters the properties of the casein micelles rendering them susceptible to aggregation and this marks the onset of secondary phase. There is no clear distinction between the end of the primary phase and the beginning of the secondary phase since the two reactions overlap to some extent with some aggregation commencing before complete hydrolysis of κ-casein (Green *et al.* 1978; Dalgleish, 1979; Chaplin and Green, 1980). This overlap of the primary and secondary phases is particularly pronounced in ultra-filtered milks.

There is, however, a critical value of κ-casein hydrolysis below which micelles cannot aggregate. Dalgleish (1979) proposed that 88% destruction of κ-casein is necessary before coagulation and this was in good agreement with Chaplin and Green (1980) who put this value at 86% κ-casein hydrolysis. The action of rennet can thus be seen as providing 'hot spots' (areas on casein micelle surfaces from which the protective GMP moiety has been "shaved off") via which the micelles can aggregate, these reactive areas being produced by removal of κ-casein from a sufficiently large area (Payens, 1979; Green and Morant, 1981; Payens, 1982). As the last of the stabilising surface is removed (i.e. during the destruction of the final 20%

of the κ-casein) the concentration of micelles capable of aggregation and the rate at which they aggregate increases rapidly. Finally, when the micelles have been completely denuded of their κ-casein macropeptide, a limiting rate for the aggregation is reached, and the micelles aggregate.

The actual mechanism of milk clotting is the least understood aspect of the renneting of milk although coagulation is the most obvious result of the effect of acid protease attack. Green *et al.* (1978) provide the most comprehensive explanation of the process of gel assembly during the secondary phase of rennet coagulation. They concluded from electron microscopic observations that the initial stages of gel formation involve the formation of small aggregates in which the micelles tend to be linked in chains rather than in clumps. The chains apparently link together randomly to form a network, which then becomes more extensive. Eventually, the network chains group together to form strands. The gel is thought to be assembled by linkage of smaller aggregates rather than addition of single particles to preformed chains. Initially linkage of aggregated micelles is through bridges, which slowly contract with time forcing the micelles into contact and eventually causing them to partly fuse. This process probably progressively strengthens the links between micelles explaining the rise in curd firmness after coagulation.

Both van der Waals and hydrophobic interactions are thought to control coagulation although the high temperature dependence of the coagulation reaction and its tendency towards dissociation at low temperatures seems to favour hydrophobic interactions as dominating (Kowalchyk and Oslon, 1977). However, because the rate of aggregation changes with the concentration of Ca^{2+}, these ions may play a part in the aggregation (Green and Marshall, 1977) which is incompatible with aggregates being held together only by hydrophobic interactions (Dalgleish, 1983). Furthermore, the rate of aggregation is decreased when the ionic strength is increased, suggesting that specific ion-pair forming may be important (Slattery, 1976;

Green and Marshall, 1977; Payens, 1977).

Tertiary phase

The tertiary stage is thought to involve processes such as syneresis and the non-specific proteolysis of the caseins in the rennet curd once formed (Dalgleish 1982).

Overall reaction

Generally, the overall reaction for the rennet coagulation of milk may be summarised as follows:

Casein → Para-casein + soluble peptides
↓
Gel (curd)

2.5.2 Factors affecting rennet coagulation

Several factors are known to influence any one or all of the phases of the rennet coagulation of milk. However, overlap of the phases has made the study of the exact effect of these factors on any one phase (particularly the secondary phase) extremely difficult. Use of immobilised enzymes has enabled some workers (e.g. Cheryan *et al.* 1975) to isolate the secondary phase from the enzymatic phase and thus provide them a unique opportunity to determine some properties of the secondary phase. Some of their conclusions are briefly discussed below along with the effects of some of these factors on the primary phase.

Influence of pH

It has been suggested that the optimum pH for the attack of chymosin on κ-casein is in the range 5.0 - 5.5 (Humme, 1972), but in milk it appears that the optimum is in the region of pH 6.0 (van Hooydonk *et al.* 1986b). The isoelectric point of κ-casein is about pH 4.5 and so measurements of

the true optimum pH are complicated by substrate precipitation below pH 5. For all proteases, hydrolysis of κ-casein is increased steadily by reducing the pH to between pH 6.7 and 5.0.

While Pyne (1955) concluded that pH only affects the primary phase, Cheryan *et al.* (1975) proposed that the contributions of the enzymic phase to the overall rate are minor compared to the effect of pH on the secondary phase. The rate of coagulation showed a marked increase as the pH of the substrate was lowered. The faster coagulation at lower pH thus reinforces the idea of coagulation being basically a charge-neutralisation process (Kowalchyk and Oslon, 1977). However, other factors must be involved since the physical properties of gels formed by milk clotting enzymes differ from those formed by isoelectric precipitation. In addition, enzymic-coagulation requires calcium whereas isoelectric precipitation does not. Green (1973), in an attempt to explain the differences in physical properties between rennet and acid gels, suggested that an additional specific interaction occurs between rennet-treated micelles in the former gels. Details of these interactions are uncertain although bridging by para-κ-casein formed on rennet treatment has been suggested (Parry and Caroll, 1969). However, Cheryan *et al.* (1975) concluded that the interactions probably involved micellar components other than or in addition to para-κ-casein.

The state of the micellar calcium phosphate (MCP) is an additional factor requiring consideration in discussing the effect of pH on rennet coagulation. It is well established that a decrease in pH leads to a decrease in rennet coagulation time (RCT) (van Hooydonk *et al.* 1986b) but most of this is probably caused by the increase in enzyme activity as the pH is lowered (Humme, 1972; van Hooydonk *et al.* 1986b). However, it has also been shown that the pH does exert some effect on the rate of coagulation of the renneted micelles (Kowalchyk and Oslon, 1977). The rate of aggregation increases as the pH decreases (Kim and Kinsella,

1989), and the extent of proteolysis required for aggregation decreases markedly (van Hooydonk *et al.* 1986b). These results, however, may depend on the method of acidification, how long the acidified milk is stored and the effect of the buffer into which the milk is diluted, since the composition of the micelles changes markedly with pH (Snoeren *et al.* 1984; Dalgleish and Law, 1989). The increase in rate of aggregation has been suggested to arise from the increase in the activity of Ca^{2+} as the pH is lowered, (Pearce, 1976), but an alternative explanation may be found by considering the effect of pH on the micellar (or colloidal) calcium phosphate (Shalabi and Fox, 1982). It has been suggested (Roefs *et al.* 1985) that dissolution of MCP may in fact lead to a decrease in the efficiency with which micelles coagulate. As the pH is lowered, more calcium phosphate dissolves, but in milk this only serves to increase the concentration of Ca^{2+}. The effects therefore tend to cancel out, to give only a small pH-dependence of the aggregation.

Influence of ionic strength

The ionic strength of the renneting medium is important in defining the activity of the proteinase. This may be because the enzyme and substrate are both negatively charged and tend to repel each other: an effect which can be overcome by increasing the ionic strength. If the ionic strength is increased too much, however, it will interfere with specific charge interactions which are essential for enzyme activity, and consequently the activity will decrease (Payens and Both, 1980; Payens and Visser, 1981).

Influence of calcium concentration

There is still some debate on the effect of Ca^{2+} on the primary phase. Some workers (Mehaia and Cheryan, 1983a; van Hooydonk *et al.* 1986a) accept that Ca^{2+} ions have no effect on the rate of conversion of κ-casein. Others (e.g. Green and Marshall, 1977), however, have found an increased rate of proteolysis after addition of calcium and other multivalent cationic additives. Yet other researchers, (e.g. Bringe and Kinsella, 1986) have

claimed that concentrations of Ca^{2+} above 8mM decrease the enzymatic activity. According to Dalgleish (1992), these different observations possibly relate to the types of experiments that were attempted, since in some the milk was diluted into a buffer solution containing Ca^{2+} (Bringe and Kinsella, 1986) while in others the solution of Ca^{2+} was added to milk (van Hooydonk *et al.* 1986a).

The secondary phase is completely dependent on a critical Ca^{2+} concentration and above this level the RCT of milk decreases with increase in the amount of calcium (Walstra and Jenness, 1984). The mechanism of the effects of calcium on the renneting process is not fully understood. Green (1982) suggested that the rate of aggregation is increased by adsorbed cations shielding the negatively charged groups of the casein and this increases the hydrophobicity of the rennet converted micelles and so promotes aggregation. Dalgleish (1983) attributed the accelerating effect of calcium to a specific interaction of unknown nature and not to a simple charge neutralisation of renneted micelles. Reduction of MCP by about 20% prevents coagulation unless the Ca^{2+} concentration is increased (Pyne and McGann, 1960) presumably because removal of MCP, which is considered to be attached through organic casein phosphate groups, increases micellar charge (Pearce, 1976).

Influence of temperature

The activity of rennet increases with temperature in the range 28 - 36°C (Phelan, 1975). However, κ-casein hydrolysis has been shown to proceed at temperatures as low as 2°C. The secondary phase, however, is extremely dependent on temperature and Q10 values (Q10 describes the temperature dependence of a reaction as a factor by which the reaction rate is changed when the temperature is changed by 10°C) ranging from 13 to 16 have been reported between 20°C and 50°C (Berridge, 1942; Ernstrom and Wong, 1974). The secondary phase is markedly retarded at temperatures <18°C.

Influence of preheat treatment of milk

In modern cheese-making practice, cheesemilk is both heated and cooled for considerable periods before use and these operations have a significant effect on the chemical nature of the milk constituents and consequently on rennet coagulation and gel formation.

Heating milk prior to renneting renders it difficult to clot by normal renneting procedures (Morrisey, 1969). The effects are insignificant until the milk has been heated to temperatures which cause the denaturation of β-Lg (Wheelock and Kirk, 1974). Several reasons for the detrimental effects of heating on rennet coagulation have been suggested by Dalgleish (1992) and include:

1. Denaturation of β-Lg and its complexing with κ-casein may cause reduced accessibility and\or susceptibility of the Phe_{105} - Met_{106} bond of κ-casein to rennet action.

2. Binding of denatured β-Lg to micelle surfaces may initiate stearic hindrance and prevent coagulation of renneted micelles.

3. Completely different and more far-reaching changes in micellar structure may be caused by heating and clotting may be hindered by neither of the first two points; perhaps heating may cause changes in the distribution of calcium phosphate in the micelles and the serum (Fox, 1981) which render even renneted micelles less capable of aggregation (van Hooydonk *et al.* 1987).

Each of these points is briefly discussed below with reference to the evidence for and against each as available in the literature.

Accessibility of the Phe_{105} - Met_{106} bond

Conflicting results are reported in the literature as to whether heat treatment affects the rate or extent of κ-casein hydrolysis in the enzymic phase or whether both of these aspects of the reaction are hindered. Hindle and Wheelock (1970), Wilson and Wheelock (1972), Wheelock and Penny (1972) and Wheelock and Kirk (1974) have carried out extensive studies on the effect of heat on the rennet hydrolysis of κ-casein in heated milk and in model systems containing casein micelles and β-Lg. They concluded that complex formation between β-Lg and κ-casein on heating was responsible for the inhibition of the hydrolysis of κ-casein due to reduced accessibility of 75% of the Phe_{105} - Met_{106} bonds of κ-casein after complete denaturation of whey proteins. Hindle and Wheelock (1970) concluded that the inhibition of the primary phase of rennet action affects mainly the release of those peptides which lack carbohydrate due to easier formation of a β-Lg\κ-casein complex with κ-casein containing less carbohydrate.

Damiez and Dziuba (1975), who applied fairly mild heat treatments (85°C for 15 s), concluded that only the rate of hydrolysis was affected but Marshall (1986) reported that the rate and the extent of conversion are hardly affected by severe heat treatments. van Hooydonk *et al.* (1987) reported a maximum reduction in the rate of hydrolysis of 25% (after heating at 95°C for 5 min) and a 10% decrease in the amount of convertible κ-casein (therefore about 10% decrease in extent of hydrolysis). Reddy and Kinsella (1990) reported that heating suspensions of casein micelles at 85°C for 15 min in the absence of whey proteins had no effect on the rennet hydrolysis but heating in the presence of β-Lg inhibited the rate and extent of hydrolysis.

Stearic hindrance of renneted micelles

This concerns hindrance of renneted micelles against coagulation in the secondary phase due to the presence of denatured whey proteins on the

micellar surface. Comparatively little attention has been given to this aspect in most research on the rennetting properties of heated milks. van Hooydonk *et al.* (1987) and Marshall (1986) have both reported failure of heated milk to coagulate following rennet addition and the former authors observed that 25% denaturation of whey proteins substantially delayed the process of aggregation and even more the rate of curd firming. More severe heat treatment (up to 75% denaturation of whey proteins) prevented formation of a gel within 3 h of incubation, although aggregation of protein particles could be observed by viscometry.

Shift in calcium equilibrium

The secondary phase of rennet coagulation is diminished in heat treated milks possibly due to shifting of the equilibrium of Ca^{2+} to the casein micelles and away from the milk serum. This is supported by the fact that the effects of heat on the renneting properties of milk may be partially or completely reversed by milk acidification and re-neutralisation (pH cycling) before rennet addition (Banks *et al.* 1987; Singh *et al.* 1988). The effect of changing the pH in this way is to partly dissociate the calcium phosphate of the micelles as the pH is lowered (Davies and White, 1960; Dalgleish and Law, 1989) followed by its reformation (and possible partial reconstruction of the micelles) as the pH is raised again, although not all of the dissociated Ca^{2+} is restored to the micelles as the pH is raised (Singh *et al.* 1988).

The effect of heating may also be reversed, at least partially, by adding $CaCl_2$, and this suggests that heating interferes with the coagulation mechanism of the renneted micelles, which is highly sensitive to Ca^{2+}, as well as with the enzymatic reaction.

The hysteresis effect

During heating, calcium phosphate is deposited on the casein micelles. Therefore, the full effect of heating on rennet coagulation will not be

observed if measurements are made immediately after heating because the newly deposited calcium phosphate accelerates the second phase of coagulation, thereby off-setting the effects of heating. However, this additional colloidal calcium phosphate redissolves on cooling (a phenomenon called hysteresis) and the full effects of heating become apparent. The β-Lg concentration in milk has been shown to be proportional to both the immediate and the hysteresis effects of heating (Kannan and Jenness, 1961). Kannan and Jenness (1961), concluded that the effect of heating (immediate) is on the primary phase while hysteresis affects the secondary stage of rennet action.

Using milk systems containing various levels of β-Lg and N-ethylmalimide, to block the β-Lg\κ-casein interaction, Morrisey (1969) confirmed that β-Lg is an important factor influencing the effect of heat on rennet coagulation. This interaction influenced both the immediate and hysteresis effects.

In summary, the β-Lg\κ-casein complex affects the primary phase by reducing the accessibility of casein to rennet (thus preventing the enzyme from hydrolysing sufficient κ-casein on the surface of the micelles to make them aggregable) and the second stage by reducing the sensitivity of the enzymatically altered casein to calcium ions. The immediate effect of heat is to increase the RCT. Little effect is observed below 72°C but the effect becomes quite marked above 80°C. The influence of heat is of course also a function of the length of exposure, moderately low temperatures being quite effective over a long period of time.

2.6 Cheese manufacture

The cheese manufacturing process may be divided into two distinct stages: manufacturing and ripening (Fox, 1993).

Milk —(manufacture)→ Fresh cheese curd —(ripening)→ Mature cheese

While the processing steps for different varieties differ in detail, the basic principles are the same and include acidification, coagulation, dehydration (cutting gel, cooking, stirring, pressing, salting and any other operation that promotes gel syneresis), and shaping (moulding and pressing).

Cheese manufacture is essentially a dehydration process in which the fat and casein in milk are concentrated between 6 and 12 fold, depending on variety. The level of moisture in the cheese is defined by the extent and combination of the above five operations and in turn the moisture and salt content as well as the cheese microflora determine the biochemical changes that occur during ripening and hence determine the flavour, aroma, and texture of the finished product. The nature and quality of the finished cheese are thus determined to a very large extent by the manufacturing steps.

Milk of high microbial and chemical quality must be selected for cheese manufacture. Since most cheese milk is now pasteurised prior to use, the chemical quality particularly as regards the milk proteins is very important since it affects rennet coagulation of milk and consequently curd tension, gel syneresis and cheese texture.

2.7 Incorporation of whey proteins in cheese

Whey proteins which account for almost 20% of the total milk proteins are not retained in the curd during traditional cheesemaking. Cheese curds therefore consist mainly of caseins which may be precipitated at low pH values and so form a gel when renneted in mildly acidic conditions.

There are several obvious benefits that may be achieved through the incorporation of whey proteins in cheese:

a) The nutritional value of the cheese is greatly enhanced.

b) Efficiency of cheese production techniques is improved resulting in higher yields.

c) The biological oxygen demand of the whey released during syneresis is reduced thus decreasing its polluting power.

Whey proteins may be incorporated in cheese either in native form or they may be denatured. Native proteins may be incorporated into the curds of soft and semi-soft cheeses through ultra-filtration. Alternatively, whey proteins may be incorporated into cheese gels in the denatured form through one of two ways:

i) The Centriwhey Process denatures whey proteins by heating the whey obtained from traditional cheesemaking and then acidifying the whey to precipitate the proteins which are then concentrated. The concentrate may then be added back to milk for cheesemaking.

ii) The milk to be used for cheese manufacture may be heat treated at temperatures sufficient to bring about denaturation.

This second technique however results in several changes in the milk system which complicate the cheesemaking process. These have been discussed in Section 2.5.2.

2.7.1 Use of ultra-filtration (UF) in cheese manufacture

Ultra-filtration (UF) is defined as a sieving process in which the constituents of milk are fractionated according to their molecular size. The pore size of the membrane determines the molecular size cut-off. There is a clear break in size between the low MW components (water, ions, and lactose) and the high MW components (fat and protein) of milk as shown in the table below:

Table 2.1:

Relative diameters of milk constituents. (From Muir and Banks, 1985)

Milk constituent	Diameter (nm)
Water	0.3
Chloride and Calcium ions	0.4
Lactose	0.8
Whey protein	3 - 5
Casein micelles	25 - 300
Fat globules	130 - 1300

This gap is exploited by commercial membranes for UF and with MW cut-offs at 20,000 - 25,000, these membranes exclude all the protein while permitting free passage of water, lactose, the non protein nitrogen (NPN) fraction of milk and the soluble salts. For milk protein separation, MW cut offs in the membrane of at least 20,000 are necessary (Renner and Abd El - salam, 1991).

Protein and fat are retained virtually complete in the concentrate. Lactose, minerals, and vitamins are fractionated between the concentrate and the permeate; the extent of this fractionation depends on the level of protein content in the final concentrate. Minerals such as calcium, magnesium, phosphorous, and citrate are present partly bound to protein in milk and partly in solution. During UF, the bound minerals are retained by the membrane and concentrated, while those in solution pass through the membrane so that a constant concentration is maintained in the aqueous phase of the concentrate (Green *et al.* 1984).

Applications of UF for cheesemaking

Cheesemaking using UF can currently be divided into three main categories (Kosikowski, 1986).

(i) *Use of protein standardised milk*: the protein content of milk collected by dairy plants varies according to season. Such a variation in the composition of the incoming milk requires adjustment of processing parameters by cheesemakers. Moreover, at low protein content, rennet curds are weak and lead to relatively high losses of caseins as fines in whey. A slight increase in the protein content by UF eliminates these difficulties. Cheese varieties successfully made using protein standardised milk include Mozzarella (Fernandez and Kosikowski, 1986), cottage (Mattews *et al.* 1976, Kosikowski *et al.* 1985; Kealy and Kosikowski, 1986) and others.

(ii) *Use of medium or intermediate concentrated concentrates*: numerous cheese varieties, ranging from soft to hard, have been made from medium concentrated concentrates. In this approach, cheese is made by using specially designed equipment able to cut and handle firm curd resulting from the coagulation of 2:1 to 5:1 concentrates, eventually diafiltered with pure salted or acidified water. The main applications in industrial operation are the manufacture of UF Cheddar cheese according to the APV - Siro Curd process (Garret, 1987) and the production of structured Feta cheese (Hansen, 1985).

(iii) *Use of liquid pre - cheese*: in this approach, cheese milk is concentrated by UF to the composition of the drained curd being made, before rennet addition. There is minimal whey drainage, and there is no need for cheese vats. This principal was first applied to Camembert cheese but many applications have been successfully developed for the manufacture of other cheese varieties, ranging

from 'fromages frais' or Quarg to semi - hard cheeses such as Saint Paulin.

Several advantages pertaining to the use of UF in cheese manufacture have been highlighted by Maubois and Mocquot (1975). These are numerically listed below.

1. Improvement in cheese yield through the incorporation of whey protein. The higher the concentration factor (CF) of the milk used, the better will be the recovery of whey proteins. There are savings in energy and manufacturing time.
2. Reduction of the quantity of rennet required for gel formation by up to 80%.
3. A reduced volume of fluid has to be processed, therefore, smaller plants are required or the plant capacity is increased.
4. UF offers potential to the manufacture of cheese from milk with a standardised content of fat, protein, and maybe lactose.
5. The simplification of the manufacturing process and the possibility of continuous processing are considered further benefits.
6. As the permeate contains no proteins and is at neutral pH, its polluting power represents only 80% of the traditional whey.
7. The nutritional value of the cheese is improved through inclusion of the whey proteins with their high biological value.
8. UF offers better control of cheese size.

The main disadvantage is that on concentrating the fat and protein, the colloidal inorganic salts are also concentrated in the same proportions as the caseins. This results in cheeses with very high mineral salts content as well as poorly developed cheese texture and flavour. This is the reason why comparatively little use of UF is currently made for the manufacture of the hard and semi - hard cheese varieties.

2.8 The rennet coagulation properties of UF concentrates

The most significant effect of UF is the increase in protein concentration and this coupled with the simultaneous decrease in the aqueous phase and increase in calcium concentration results in major influences on the rennet coagulation properties of UF concentrates.

It is generally agreed (Garnot and Corre, 1980; Garnot *et al.* 1982; Mehia and Cheryan, 1983b) that concentration of milk results in a decrease in the degree of κ - casein proteolysis during the enzymic phase. However, aggregation commences at a lower degree of proteolysis (about 50% depending on the volume concentration ratio - VCR) than that observed in unconcentrated milk (80 - 90%). The primary reaction does, however, continue slowly to completion after gelation at a lower degree of proteolysis. The degree of proteolysis at coagulation is also dependant on pH and the type of enzyme used.

Researchers are divided in their views as to the influence of UF on the (rennet) coagulation time of milk. Some workers (e.g. Green and Morant, 1981; Dalgleish, 1982; Reuter *et al.* 1981; Darling and van Hooydonk, 1981; Mehaia and Cheryan, 1983b; Lucisano *et al.* 1985) have reported a reduction in coagulation time of milk after UF. Others (e.g. Garnot and Corre, 1980; Chaplin and Green, 1980; Dalgleish, 1982; Garnot *et al.* 1982; Payens, 1984) obtained an increase in coagulation time after UF. Dalgleish (1981) has also reported that UF concentration has no effect on the coagulation time of milk. It is evident that the different methods used to evaluate coagulation time, the various experimental conditions (e.g. type of enzyme used and pH at renneting), mechanical handling of milk and precision and sensitivity of the methods adopted to detect the beginning of the coagulation process, contribute to these discrepancies.

It is generally accepted that there is a steep increase in the rate of gelation and final gel strength following renneting of UF treated milks (Schmutz

and Puhan, 1978; Dalgleish, 1980; Green *et al.* 1981 and Payens, 1984). Schmutz and Puhan (1978) attributed the increased rate of gel formation and final gel strength to increased protein concentration, reduced pH (pH 6.53 at 10.2% casein, pH 6.6 at 2.8%) and increased total as well as ionised calcium.

2.8.1 Rennet curds formed from UF concentrates

During UF, the colloidal calcium concentration increases roughly maintaining a constant ratio with the protein concentration. Together with the increased casein concentration, the modified calcium equilibrium is certainly a major cause of the increased firmness and elasticity of curds obtained from UF concentrates.

These curds are different from the conventional rennet curd with respect to their structure. In particular, the firmness of these curds differs essentially as the protein concentration is much higher and the higher the protein concentration, the firmer the coagulum. At high protein concentrations, UF curds have proved too strong and too coarse for traditional hard cheese manufacture. To reduce these effects and to obtain curds more similar in composition and rheology to the traditional products, an attempt was made to decrease the calcium concentration or, more specifically, the colloidal calcium - to - protein ratio by applying UF after acidification of milk or by diafiltration treatments (Covacevich and Koskowski, 1977; Lucisano and Casiraghi, 1985). These techniques are likely to shift the calcium from the colloidal to the soluble form, which is then removed with the permeate during UF. Casiraghi *et al.* (1987) demonstrated that curd firmness was directly related to ionic calcium concentration and was therefore increased by pH reduction and reduced by citrate addition. However, in all cases, the concentrates yielded firmer curds than milk, and the firmness increased with time after rennet addition.

Generally, UF concentrates have excellent properties which make it possible, to a much higher degree than for normal milk, to form a firm curd and easier to cut coagulum. This will probably offer important new possibilities for future cheese technologies in relation to yield and\or water binding.

Since gel formation commences in UF concentrates when a smaller proportion of the micellar κ - casein has been hydrolysed, micelles that are not sufficiently modified at the point of coagulation presumably become incorporated into the gel later but probably do not form an integral part of the gel structure (Dalgleish, 1981). Gels prepared from concentrates become progressively coarser as the protein concentration of the milk increases; the less developed structure of the curd tends to make it fragile (Lelievre and Lawrence, 1988).

2.9 Heat treatment of UF concentrated milks

Although some studies have been reported in which milk was concentrated by evaporation (McKenna and O'Sullivan, 1971) or in which whey powder was reconstituted to various levels of total solids (Hillier *et al.* 1979), very little information is available in the literature concerning the effect of heat treatment of UF concentrated milks on whey protein denaturation and association with casein micelles.

McMahon *et al.* (1993) observed significantly greater whey protein denaturation in (UF treated) 3X (VCR) UHT milk (72%) than in 1X UHT milk (62%). This was essentially in agreement with Dargan and Savello (1990) who reported more whey protein denaturation in UF milk concentrated to 5% protein than in milk fortified to 5% protein by addition of skim milk powder. Green (1990) also observed more whey protein denaturation in UF concentrated milks than in normal milk after heat treatment at temperatures in the range 90 - 97°C for 15 s. McMahon *et al.* (1993) concluded that the influence of heat treatment of milk on whey

protein denaturation is dependent on the method of concentration used. In UF concentrated milks, the non-protein solids do not increase in proportion to protein concentration. As such, in UF milk there is no protective effect offered (by lactose) to the whey proteins against denaturation and so these are more susceptible to denaturation in UF milks. McMahon *et al.* (1993) further reported that more protein material adhered to the casein micelles after heat treatment of 3X UF milks as compared to normal milk. They attributed this to the higher whey protein denaturation in the UF concentrates as well as the availability of less water for dispersion of casein micelles in concentrated milk.

2.9.1 Influence of heat treatment on rennet coagulation properties of UF concentrated milks

Sharma *et al.* (1990) studied the effect of heat treatment of UF milk on its coagulation properties. They reported that heat treatment (85°C for 15 min.) increased coagulation times (27% in 4X, 31% in 3X and 100% in 2X UF concentrates) compared to normal milk which failed to coagulate. In all samples, the rate of gel formation and final gel strengths were decreased as the heating temperature became more severe.

McMahon *et al.* (1993) also studied the influence of heat treatment on rennet coagulation properties of UF concentrated milks. They reported that skim milk heated to 140°C did not coagulate within 2 h. UHT processed 3X UF concentrate did coagulate although the curd firmness was much lower than in unheated 3X concentrates. There was, however, less observed difference in final gel firmness as a function of process temperature up to 123°C.

CHAPTER 3

OBJECTIVES

(1) To determine the influence of heat treatment of skim milk on:
   (i) denaturation of whey proteins
   (ii) association of denatured whey proteins with casein micelles
   (iii) incorporation of denatured whey proteins into rennet gels
   (iv) rennet coagulation properties.

(2) To relate the denaturation/aggregation of whey proteins to the rennet coagulation properties of the milk.

(3) To investigate methods of improving the rennet coagulation properties of heated skim milk.

(4) To determine the influence of UF and heat treatment on the rennet coagulation properties of skim milk.

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials

4.1.1 Milk source

Bulk raw milk was obtained from Massey University Dairy Farms in 50 litre containers. Town milk was used since this shows less variation in composition than seasonal manufacturing milk (particularly the protein content) throughout the year.

4.1.2 Rennet source

Standard calf rennet which contains 59 RU/ml, was obtained from The New Zealand Rennet Company (Eltham, New Zealand).

4.2. Processing methods

4.2.1. Preparation of skim milk

Whole milk was transferred into 20 litre containers and warmed to 45 - 50°C in a water bath and the fat removed using a pilot-scale centrifugal separator (Alfa-Laval; model 103 AE, Hamilton, New Zealand). The resultant skim milk was immediately cooled in a cold water bath and used in subsequent experiments.

4.2.2. Concentration of skim milk

Samples of skim milk were warmed to 45°C and then concentrated to volume concentration ratios (VCR) of either 2X or 3X (the VCR is defined as the initial volume of milk divided by the retentate volume) using the Protosep III KOCH-UF pilot plant (Massey Food Technology Department Pilot Plant, Palmerston North, New Zealand). A HFM-100 membrane of area 0.28m^2 and molecular weight cut off 30,000 was used.

The operating conditions used were as follows: the product flow-rate was approximately 1400 litres per hour, the operating temperature was 50°C, the inlet pressure was 240 kPa and the outlet pressure was 160 kPa.

4.2.3. Heat treatment of skim milk and UF concentrates

The skim milk and the UF concentrates were heated at temperatures in the range 80 - 140°C using a mini UHT plant (Spiral flow indirect UHT plant: Alfa-Laval, Australia). The UHT plant used indirect heat exchange through a spiral heat exchanger to bring the samples to the required processing temperature. Milk and UF concentrate samples were held at the processing temperature for 4 s (excluding come-up time), after which they were cooled regeneratively to below 30°C.

4.3 Analytical methods

4.3.1 Total solids

Skim milk (5g) was weighed into a flat bottomed flask and heated for 3 h in an air oven at 100°C. After cooling and weighing, the % residue was reported as the total solids content of the milk (method 16.032, AOAC methods 1980).

4.3.2 Milk protein concentration

The protein concentration was estimated by determining the total nitrogen content by the Kjeldahl method, using a Kjeltec 1026 system (Tecator, Sweden). The total protein contents were calculated from the nitrogen percentage by multiplying by an empirical factor of 6.38.

4.3.3. Polyacrylamide gel electrophoresis (PAGE)

Theory

Electrophoresis exploits the amphoteric nature of proteins which enables them to migrate after application of an electric field. This migration is

influenced by the pH and differs for most proteins due to differences in their net charges and molecular weights. Therefore, if an electric field is applied to a mixture of proteins, they migrate at different rates dependant on their charge-to-mass ratio.

For the analysis of residual native proteins, Native-PAGE was used. This uses a non-dissociating and non-reducing buffer system and so only allows the analysis of those proteins still in their native monomeric form.

The ionic detergent, sodium dodecyl sulphate (SDS), and a thiol reagent, 2-β mercaptoethanol, were used to create the dissociating and reducing buffer system respectively in SDS-PAGE. In this buffer disulphide bonds are cleaved (due to 2-β-mercaptoethanol) and SDS which binds to the proteins in a constant weight ratio (1.4g SDS/g protein) disrupts the non-covalent interactions between protein molecules. The intrinsic charges of the protein are insignificant compared to the negative charges provided by the bound detergent so that SDS-protein complexes have essentially identical charges and migrate in gels strictly according to protein size. SDS-PAGE, therefore, allows for analysis of the total protein content in the samples, irrespective of whether these were in the denatured or native state.

The procedure used for SDS-PAGE was essentially the classical method of Laemmli (1970), as described by Singh and Creamer (1991a) while Native-PAGE was run as described by Andrews (1983).

Native-PAGE

Preparation of solutions

Acrylamide / Bis (30% T, 2.67%C)

Acrylamide (30g) and N,N-bis acrylamide (0.8g) were dissolved in deionised water to give a final volume of 100ml. The solution was stored in a dark

bottle at 4°C.

Resolving gel buffer

TRIS (Trishydroxymethylaminoethane, 36.3g) was added to 90ml of deionised water. The pH was then adjusted to 8.8 with 6M HCl before the solution was brought to 200ml with deionised water and stored at 4°C.

Stacking gel buffer

TRIS (6.0g) was dissolved in 60ml deionised water. The pH was then adjusted to 6.8 with 1M HCl and the solution brought to 100ml with deionised water. The buffer was stored at 4°C.

Sample buffer

500ml of sample buffer was prepared by mixing 100ml of stacking gel buffer with 300ml of deionised water, 10ml 0.10% bromophenol blue and 40ml glycerol. The pH was checked to be 6.8 and the buffer stored at 4°C.

Electrode buffer

TRIS (7.5g) and glycine (36.0g) were added to about 400ml deionised water. The pH was adjusted to 8.3 after which the solution was made up to 500ml with deionised water. For each electrophoresis run, 80ml of this stock solution was diluted with 320ml deionised water.

Preparation of gel

For preparation of 20ml resolving gel, the following quantities of solutions were mixed: acrylamide/bis solution (10ml), resolving gel buffer (2.5ml) and deionised water (7.5ml). The gel solution was degassed for 15 min at 20°C with continuous stirring. 10µl of TEMED (N,N,N′,N′ tetramethylenediamine) and freshly made 10% (w/v) ammonium persulphate (100µl) were added. The gel solution (3.3ml) was then poured between two electrophoresis glass casting plates (Bio-Rad Mini Protean, Bio-Rad, Richmond, CA, USA). Approximately 400µl of deionised water

was poured over the gel solution which was then left to polymerise at 20°C. The water was drained and the polymerised resolving gel dried with filter paper before pouring the stacking gel. The stacking gel was prepared by adding 1.25ml of the acrylamide/bis stock solution to 2.5ml stacking gel buffer and 6.25 ml deionised water. After degassing (15min/20°C), TEMED (10µl) and 10% ammonium persulphate (50µl) solution were added and carefully mixed prior to applying the solution to the top of the resolving gel and inserting a 10 slot comb.

Gel electrophoresis

Two gels were fitted into a Mini-Protean II Slab Electrophoresis System (Bio-Rad, Richmond, CA, USA). 400ml of electrode buffer was then used to completely fill the inner buffer chamber and partially fill the outer buffer chamber. 100µl of the sample to be analysed was then dispersed in 900µl of Native-PAGE sample buffer (1:10 dilution) and thoroughly mixed using a vortex mixer. Using a syringe, 10µl of the diluted sample was then applied to the wells in the gels. Electrophoresis was run at 200V for ~1 h.

Staining

After electrophoresis, the gels were stained in ~50ml Coomasie brilliant blue R solution (1g brilliant blue R was dissolved in 500ml of isopropyl alcohol and 200ml acetic acid and the contents made to 2 litre with distilled water) for 2 h.

Destaining

The staining solution was replaced with destaining solution (100ml isopropyl alcohol and 100ml acetic acid diluted to 1 litre with distilled water) and the gels destained for 1 h. After 1 h, the solution was replaced and destaining continued for 19 h. Both staining and destaining were done in sealed containers on a rocking tray to ensure uniform staining and destaining on the gels.

Densitometry

Quantitative estimation of the protein resolved by Native-PAGE was made by densitometry using a laser densitometer (LKB Ultrascan XL, LKB Produkter, AB Sweden). In the densitometer, the protein bands on the stained gel were scanned by a narrow beam of laser light and the absorbance at 522nm plotted as a function of track distance. The output from the densitometer was quantified by measuring the areas under the individual peaks. The scanning procedure involved cleaning the densitometer gel plate, placing the gel on the densitometer gel plate, defining the tracks and scanning each individual band. Results were plotted as a graph of individual peaks and a table of individual peak areas prepared by a printer attached to the densitometer.

SDS-PAGE

Preparation of stock solutions

Acrylamide / Bis (30% T, 2.67% C)

This was prepared as described for Native-PAGE.

1.5M TRIS-HCl buffer

TRIS (18.15g) was dissolved in approximately 60ml deionised water. The pH was adjusted to 8.8 using 6M HCl and the volume made up to 100ml with deionised water and stored at 4°C.

0.5M TRIS-HCl buffer

TRIS (6g) was dissolved in approximately 60ml deionised water. The pH of the solution was then adjusted to 6.8 with 6M HCl and the volume made up to 100ml with deionised water and the solution stored at 4°C.

10% SDS solution

10g sodium dodecyl sulphate (SDS) was dissolved in 50ml of deionised water with gentle swirling. After complete dissolution of the SDS, the

solution was made up to 100ml with more deionised water.

5X SDS electrode buffer

The electrode buffer was prepared by dissolving TRIS base (9.0g), glycine (43.2g) and SDS (3.0g) in 400ml of deionised water. The volume was then brought to 600ml with more water and the pH checked to be 8.3. For each electrophoresis run, 60ml of this stock solution was diluted with 240ml of deionised water.

Preparation of gel

The resolving gel was prepared by mixing the following proportions of solutions: deionised water (2.02ml), 1.5M TRIS-HCl buffer (2.5ml), 10% SDS solution (100µl) and acrylamide/bis solution (5.3ml). After degassing (15min/20°C), TEMED (5µl) and 50µl ammonium persulphate (10% freshly made) were added. The gel solution (3.3ml) was left to polymerise as described for Native-PAGE. The stacking gel was prepared by mixing deionised water (6.1ml) with 0.5M TRIS-HCl buffer (2.5ml), 10% SDS solution (100µl) and acrylamide/bis solution (1.3ml). The solution was degassed (15min/20°C) and polymerisation initiated by addition of TEMED (10µl) and ammonium persulphate (50µl). The stacking gel was poured between the gel plates and the comb inserted as in Native-PAGE.

Gel electrophoresis

The gels were run as in Native-PAGE with the Native-PAGE electrode buffer being replaced with SDS-PAGE electrode buffer.

Staining, destaining and densitometry

These were all carried out as described for Native-PAGE.

4.4 Experimental procedures

4.4.1 Residual native and non-sedimentable whey proteins

Sample preparation

To determine the effects of heating temperature on the denaturation of whey proteins in skim milk or UF concentrates and their association with casein micelles, sub-samples were obtained from the bulk samples that had been heated as described in section 4.2.3. Each sub-sample (10ml) was placed in a centrifuge tube and ultra-centrifuged at 100,000*g* for 1 h (Beckman ultra-centrifuge model L2-65B: Beckman Instruments California, USA). The resultant supernatants were then analysed for residual native whey proteins (Native-PAGE) and residual total non-sedimentable whey protein (SDS-PAGE).

Residual native whey protein

The amount of residual native whey proteins in each sample was determined from the areas of the peaks on the densitometric scans obtained from Native-PAGE and expressed as a percentage of the original amount of native proteins in an unheated control. The amount of denatured whey protein could thus be derived.

Non-sedimentable whey protein

The amount of non-sedimentable whey protein in each sample was determined from the areas of the peaks on the densitometric scans obtained from SDS-PAGE and expressed as a percentage of the original amount of non-sedimentable whey protein in an unheated control. By inference, the amounts sedimented with and presumably associated with the casein micelles could be estimated.

4.4.2. Rennet coagulation properties

Renneting

A sample of milk or UF concentrate (100ml) was adjusted to pH 6.5 with 1M HCL or 1M NaOH and equilibrated at 32°C for 30 min in a water bath. It was then thoroughly mixed with 0.16ml of diluted rennet (1:10) for 30 s. This final rennet concentration of 0.16ml/L of milk was chosen since this is the normal level used in cheesemaking in New Zealand.

In some instances, calcium chloride was added (at concentrations of 1 or 2mM) to the skim milk 10 min prior to rennet addition at 32°C.

pH cycling

Samples of skim milk or UF concentrates were acidified to pH 5.5 with 1M HCl, held for 2 h at room temperature, and then reneutralised to pH 6.5 with 1M NaOH as described by Singh *et al.* (1988). This process has been referred to as pH cycling.

For heated and unheated milks, this process was carried out immediately prior to renneting while for the heated concentrates it was carried out either after concentration and heating immediately before renneting or after heating and before concentration of the milk (for details see Chapter 7).

Dynamic measurement of rennet coagulation properties

The viscoelastic properties of the renneted milks and concentrates were recorded continuously as a function of time in a controlled strain Bohlin VOR Rheometer (Bohlin Rheologi, Lund, Sweden.) using low amplitude oscillation as described by Bohlin *et al.* (1984). Immediately after the addition of rennet to the milks (this took about 40 s), 13ml milk was placed between two coaxial cylinders which make up the rheometer measuring cell. During measurement the renneted milk was subjected to a harmonic,

low amplitude shear strain, γ, of angular frequency ω

$$\gamma=\gamma_o \cos \omega t,$$

where γ is shear strain, γ_o strain amplitude, ω angular frequency (i.e. $2\pi\nu$), ν oscillation frequency, t is time in s and cos ωt is a simple harmonic function. The shear strain results in an oscillation shear stress, σ, of the same angular frequency but which is out of phase by the angle δ.

$$\sigma = \sigma_o \cos (\omega t+\delta).$$

The storage (elastic) modulus, G′, of the coagulating milk system was measured as a function of time from rennet addition. G′, which represents the curd elasticity or firmness (Guinee *et al.* 1994), is given by the equation

$$G' = (\sigma_o/\gamma_o)\cos \delta.$$

To ensure that measurements were in the viscoelastic region, a low amplitude shear strain of 0.07215, was applied at a frequency of 1 Hz. Measurements, which started 40 s after rennet addition, were taken over a period of either 1 h or 2 h depending on the experiment.

All the parameters related to the rheology of the forming milk gel were recorded using software in a personal computer connected to the Bohlin. These include the storage modulus, G′, loss modulus, G″, complex modulus, G*, phase angle, δ, time, temperature, viscosity, frequency, range, amplitude, and a correction factor which were automatically plotted during each run.

From these parameters, the gelation time (GT), the gel strength (G′), and the rate of curd firming (K) could be determined.

The GT, which marks the transition of the milk from a sol to a gel, was evident from the sudden decrease of the phase angle from 90° (for viscous fluid -i.e milk) to around 30 - 40°. As the rennet gel developed, there was a further drop (gradual) to about 15° which is characteristic of the milk gel.

The storage modulus G′ was used as an indication of gel strength at any given time while the slope of the G′ vs time curve (obtained through linear regression) was used to estimate the rate of curd firming (K).

4.4.3 Whey protein incorporation into skim milk rennet gels

Milk samples (100ml) were renneted as described in section 4.4.2 and maintained at 32°C for 2 h. The resultant milk gels were then transferred to centrifuge bottles and centrifuged at about 10,000*g* for 40 min (Sorvall SS-3 Automatic centrifuge -Du Pont instruments, Connecticut). The supernatant was filtered using Whatman number 1 paper before being analysed for residual whey proteins using SDS-PAGE. Whey protein detected in the rennet whey was taken as the non-incorporated portion and expressed as % of original. By inference, the amount of whey protein incorporated into the rennet gel could be estimated.

CHAPTER 5

INFLUENCE OF THERMAL DENATURATION AND AGGREGATION OF WHEY PROTEINS ON RENNET COAGULATION OF SKIM MILK

Whey proteins may be incorporated into cheese in denatured form as a result of heat treatment of the milk before renneting. However, it is well established that milk which has been heated at temperatures >70°C has a longer coagulation time, and forms a weaker curd than the original unheated milk. This effect is generally believed to arise from the formation of complexes between denatured β-lactoglobulin (β-Lg) and κ-casein of the casein micelles (Wheelock and Kirk, 1974; van Hooydonk *et al.* 1987; Singh and Fox, 1987b; Singh *et al.* 1988). This interaction alters the micellar surface in such a way as to make it difficult for two renneted micelles to approach sufficiently close as to coagulate. Alternatively, the complex may alter the conformation of the κ-casein so as to render it less susceptible to attack by rennet, or the attached β-Lg may simply sterically hinder the approach of the enzyme.

To date, most of the investigations on the effects of heat treatment of milk on renneting have not attempted to relate heat-induced changes in milk to changes in rennet coagulation. Dalgleish (1990a) made quantitative studies on the effect of whey protein denaturation on the rennet coagulation time of milk. No studies have been reported on the quantiative effects of the denaturation and aggregation of whey proteins on renneted micelle aggregation and gel formation.

This chapter describes the influence of heat treatment of skim milk on the secondary phase of renneting (i.e. onset of gelation and development of gel structure). Relationships between denaturation and aggregation of whey proteins and rennet coagulation properties have also been described.

5.1 Whey protein denaturation and association with casein micelles

Milk samples were heated at temperatures between 80 and 140°C for 4s. Sub-samples were ultra-centrifuged at 100,000*g* for 1 h and the supernatants were analysed using Native-PAGE and SDS-PAGE as described in Chapter 4.

5.1.1 Effect of heating temperature

Whey protein denaturation

Figure 5.1 shows the quantitative analysis obtained by scanning the protein bands obtained after Native-PAGE. The results presented are typical of the trend observed on analysing three different samples of bulk milk. The amount of native β-Lg decreased gradually as the heating temperature was increased from 80 to 120°C. Further increase in heating temperature up to 140°C resulted in little further change. α-La was less sensitive to heat treatments than β-Lg. The quantity of native α-La decreased slowly with temperature and ~50% native α-La could still be detected after a heat treatment of 140°C for 4s.

Association of whey proteins with casein micelles.

It was assumed that any denatured whey proteins associated with the casein micelles would be sedimented along with the micelles upon ultracentrifugation (100,000*g* for 1h). It must be pointed out, however, that large aggregates of β-Lg may also become sedimented during ultracentrifugation and cannot be distinguished from the β-Lg associated with the casein micelle. The formation of β-Lg aggregates probably involves interactions between denatured β-Lg molecules and it is also likely that the denatured β-Lg molecules associated with the casein micelles will further associate with denatured β-Lg. Both phenomena are therefore connected and quantitative results are given as fraction (%) of β-Lg sedimented with the casein micelles. Any β-Lg detected in the ultracentrifugal supernatants is either in native form or complexed with other whey proteins or serum caseins.

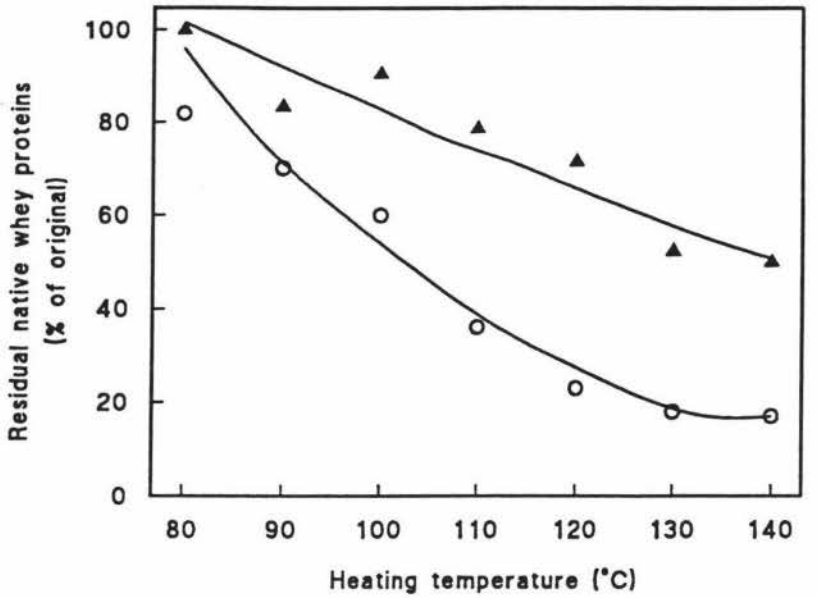

Figure 5.1: Loss of native β-Lg (o) and α-La (▲) from the ultracentrifugal supernatants (100,000*g* for 60 min) obtained from skim milk heated at temperatures in the range 80 - 140°C for 4s (estimated using quantitative Native-PAGE).

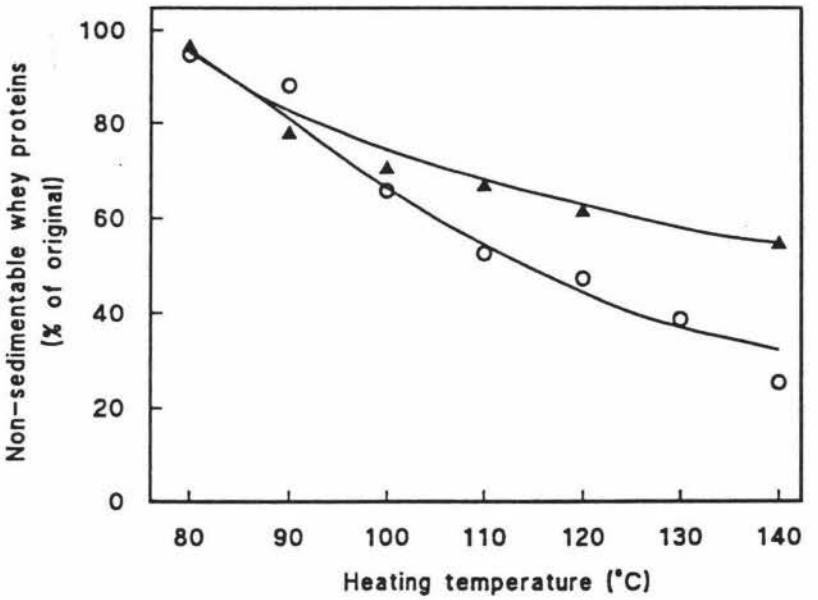

Figure 5.2: Changes in the amounts of total β-Lg (o) and α-La (▲) (native and aggregated) remaining in the ultracentrifugal supernatants obtained from skim milk heated at temperatures in the range 80 - 140°C for 4 s (estimated using quantitative SDS-PAGE).

Figure 5.2 shows that there was a decrease in the amount of non-sedimentable β-Lg as the heating temperature was increased. Heating temperatures between 80 and 110°C resulted in a rapid decrease in the amount of non-sedimentable β-Lg. Heat treatments >110°C caused a further decrease in non-sedimentable β-Lg. The extent of sedimentation of α-La was lower than that of β-Lg. The amount of non-sedimentable α-La decreased gradually between 80 and 100°C, but changed only slightly between 100 and 140°C. About 55% of the original α-La still remained non-sedimentable.

Relationship between β-Lg denaturation and association with casein micelles

Figure 5.3 compares the loss of native β-Lg resolved on native-PAGE with the loss of non-sedimentable β-Lg resolved on SDS-PAGE (Figure 5.1 versus Figure 5.2- note that Figure 5.3 should be read from right to left). It is apparent that up to 30% denaturation of β-Lg caused relatively little (~10%) association between β-Lg and casein micelles. The extent of association increased rapidly (from ~10% to ~35%) between 30% and 40% denaturation of β-Lg. Further denaturation (up to ~80%) of β-Lg resulted in a small increase in the extent of association.

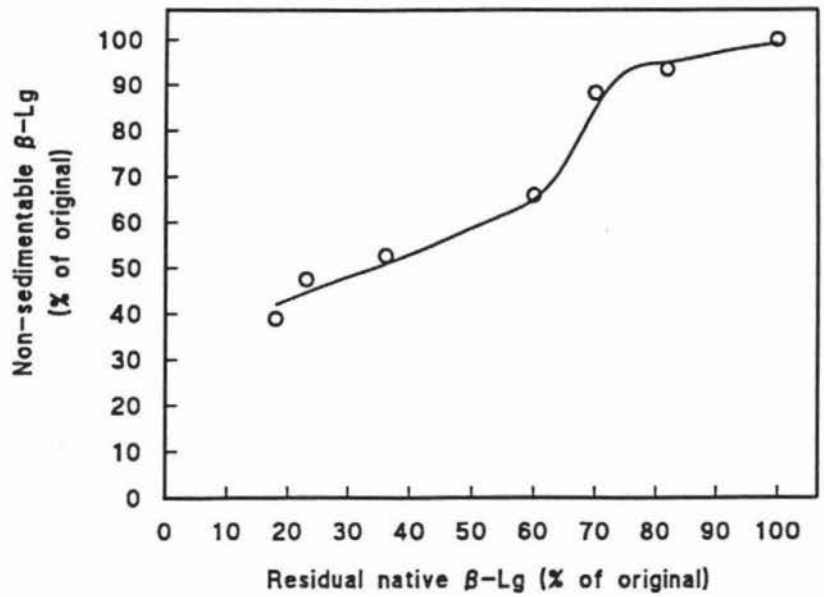

Figure 5.3: Relationship between loss of native β-Lg (from Figure 5.1) and the changes in the amounts of non-sedimentable β-Lg (from Figure 5.2).

The order of heat resistance of whey proteins found in this study was α-La > β-Lg (A>B) > BSA (results not shown), which is in agreement with the results of Larson and Rollerri (1955), Hillier (1976), Dannenburg and Kessler (1988) and Singh and Creamer (1991b). The degree of denaturation of β-Lg at different temperatures was comparable to the results reported by Law *et al.* (1994).

The formation of a complex between β-Lg and κ-casein of the casein micelles in heated milk has been well established (Morr, 1973; Snoeren and van der Spek, 1977; Creamer *et al.* 1978); hydrophobic interactions and thiol-disulphide interchange reactions appear to underly this interaction (Smits and van Brouwershaven, 1980; Singh and Fox, 1987b; Haque and Kinsella, 1988). When milk is heated, α-La shows little association with κ-casein (Wilson and Wheelock, 1972; Elfgam and Wheelock, 1977) although it has been reported to form intermolecular complexes with β-Lg, presumably *via* sulphydryl-disulphide interchange reactions (Hunzikier and Tarassuk, 1965; Shalabi and Wheelock, 1976). However, Noh *et al.* (1989) found no evidence for any specific α-La/β-Lg

complexes in heated milk.

The results presented here suggest that the association of whey proteins with casein micelles is a much slower process than their thermal denaturation, which is essentially in agreement with the data of Singh and Creamer (1991b). The data presented in Figure 5.3 indicates that there are possibly at least two different steps which may be involved in the complex formation between β-Lg and κ-casein. In the first step changes in β-Lg occur which do not involve κ-casein while the second step involves interactions of β-Lg with κ-casein. The lag phase shown in Figure 5.3 (where there is little change in non-sedimentable β-Lg) may correspond to the first step during which β-Lg either self-aggregates or aggregates with α-La and other whey proteins. In the second step, the aggregated β-Lg may then interact with κ-casein (and possibly other casein proteins) on the micellar surface. This second step appears to be initiated only when ~35% of the β-Lg has been denatured (Figure 5.3).

5.1.2 Effect of pH at heating.

Whey protein denaturation

The extreme heating conditions (140°C for 4s) employed in this experiment resulted in virtually complete denaturation of all whey proteins except α-La. As a result, very faint β-Lg bands were obtained on Native-PAGE and any differences in the extent of denaturation of this protein as a result of the variations in pH at heating could not be accurately determined. The denaturation of α-La was not significantly influenced by pH at heating.

Association of whey proteins with casein micelles

Figure 5.4 shows the amounts of non-sedimentable β-Lg and α-La in milk samples heated at 140°C for 4s at pH values in the range 6.4 to 7.3.

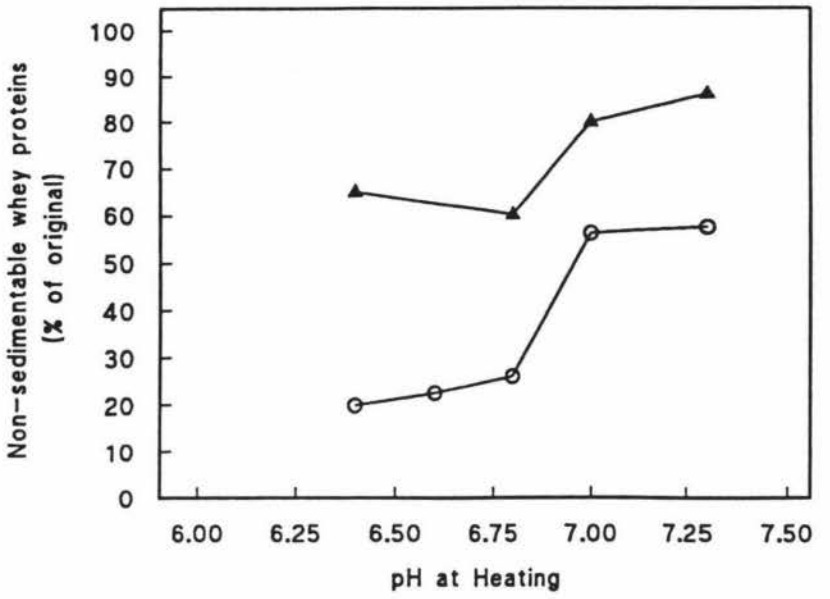

Figure 5.4: Influence of pH at heating on the amounts of total β-Lg (o) and α-La (▲) (native and aggregated) remaining in the ultracentrifugal supernatants (estimated using quantitative SDS-PAGE). Heat treatment was at 140°C for 4 s.

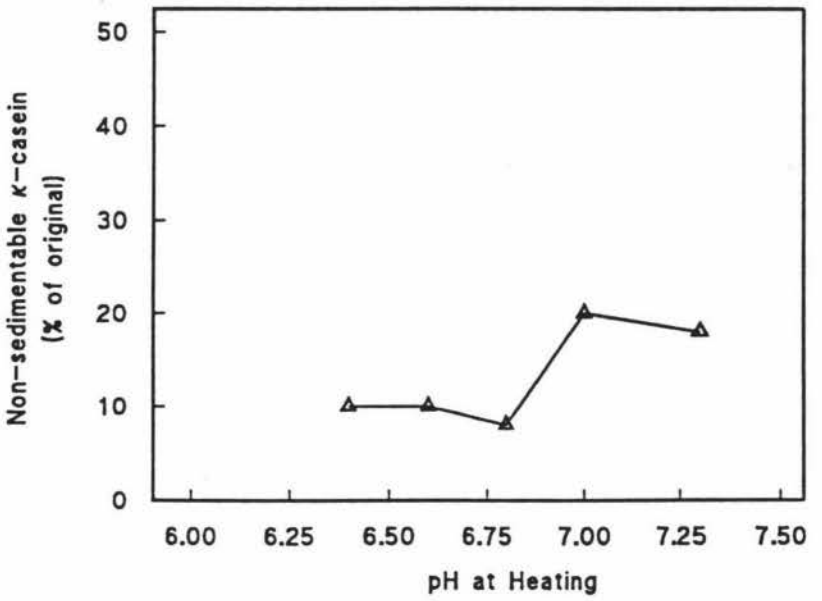

Figure 5.5: Influence of pH at heating on the formation of non-sedimentable κ-casein (estimated using quantitative SDS-PAGE) on heating skim milk at 140°C for 4 s.

As the pH at heating was increased from pH 6.4 to pH 6.8, there was a slight increase in the amount of non-sedimentable β-Lg. Above pH 6.8, however, there was a marked increase. Approximately 55% of the total β-Lg remained non-sedimentable at pH ≥7.0 compared with ≤25% at pH ≤6.8. The amount of non-sedimentable α-La showed little change on heating at pH values <6.8, but there was a marked increase upon heating at pH ≥7.0.

Dissociation of micellar κ-casein

Figure 5.5 shows the effect of pH at heating on the formation of non-sedimentable κ-casein. The concentration of non-sedimentable κ-casein, obtained from SDS-PAGE, showed little change after heating at pH between 6.4 and 6.8. However, at pH 7.0 and 7.3, its concentration increased markedly, indicating the dissociation of micellar κ-casein into the serum on heating at elevated pH.

The above results suggest that on heating milk at low pH (≤6.7), more whey proteins attach onto the casein micelles than heating at higher pH values (>6.7). This conclusion is essentially in agreement with the results of Creamer and Matheson (1980), Smits and van Brouwershaven (1980) and Singh and Fox (1987b).

β-Lg exists as a dimer at low temperatures and dissociates to monomers at 30°C. At 65°C, it begins to unfold, leading to increased reactivity of its -SH group (Sawyer, 1969). The reactivity of the -SH group of β-Lg also increases markedly at pH values >6.8 due to the N⇌R conformational change (Dunill and Green, 1966) and therefore disulphide-linked complex formation between β-Lg and κ-casein should be enhanced at high pH values. Singh and Fox (1987b) showed that sulphydryl-disulphide interchange reactions between casein micelles and β-Lg occur even at pH 7.3. At pH ≤6.8, interaction between β-Lg and κ-casein prevents the dissociation of micellar κ-casein but at pH values >6.8, the β-Lg/κ-casein complex dissociates from the micelles (Singh and Fox, 1987b). This

explains the observation of increased β-Lg and κ-casein concentrations in the ultracentrifugal supernatant (Figures 5.4 and 5.5).

5.2 Incorporation of whey proteins into rennet gels

To measure the amount of whey proteins incorporated into rennet gels, milk samples heated at temperatures in the range 80 - 140°C, were renneted as described in Chapter 4. After 2 h incubation, the gels formed were centrifuged at 10,000*g* for 40 min. The supernatant (rennet whey) was analysed for residual whey proteins using SDS-PAGE and results expressed as % of the unheated control.

5.2.1 Effect of heating temperature

The effect of heat treatment of skim milk on the amount of residual whey proteins in rennet whey is shown in Figure 5.6. Heating at 80°C had very little effect on the amounts of residual β-Lg and α-La in the rennet whey. There was a sharp decrease in the residual β-Lg on heating between 80 and 120°C. Higher temperature treatments resulted in little further decrease in residual β-Lg; a value of about 30% residual β-Lg (i.e. 70% incorporation) after heating at 140°C was observed.

The amounts of α-La incorporated into rennet gels were lower than those of β-Lg. Incorporation of this protein occurred at a steady rate between 80 and 120°C, as shown by the decrease in the amount of residual α-La in the rennet whey. Temperatures above 120°C caused little further incorporation. The minimum amount of residual α-La detected using the heating temperature-time profiles employed was about 60% at 140°C which corresponds to about 40% incorporation.

The results presented above are essentially in agreement with Law *et al.* (1994) who reported that significantly more β-Lg than α-La was incorporated into Cheddar Cheese manufactured from heated milk (90°C for 30 s).

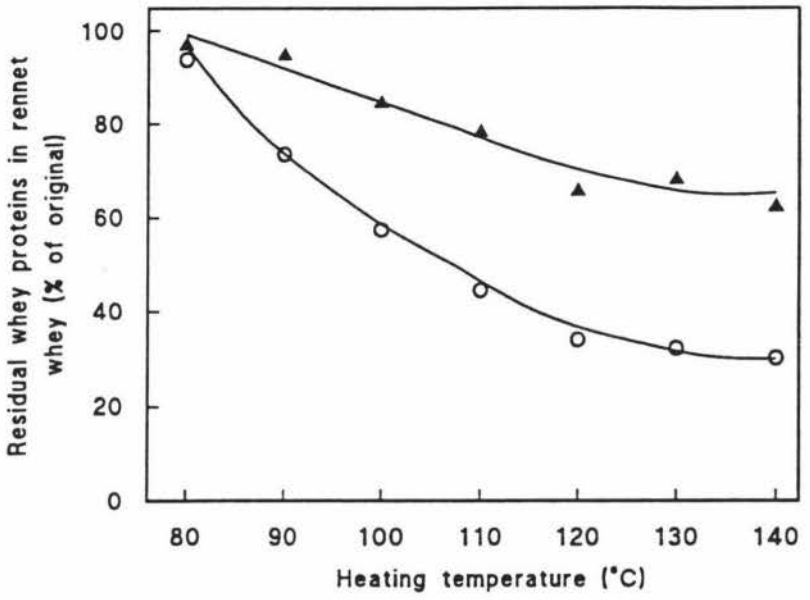

Figure 5.6: Changes in the amounts of residual β-Lg (o) and α-La (▲) in rennet whey obtained from skim milk heated at temperatures in the range 80 - 140°C for 4 s (estimated using quantitative SDS-PAGE).

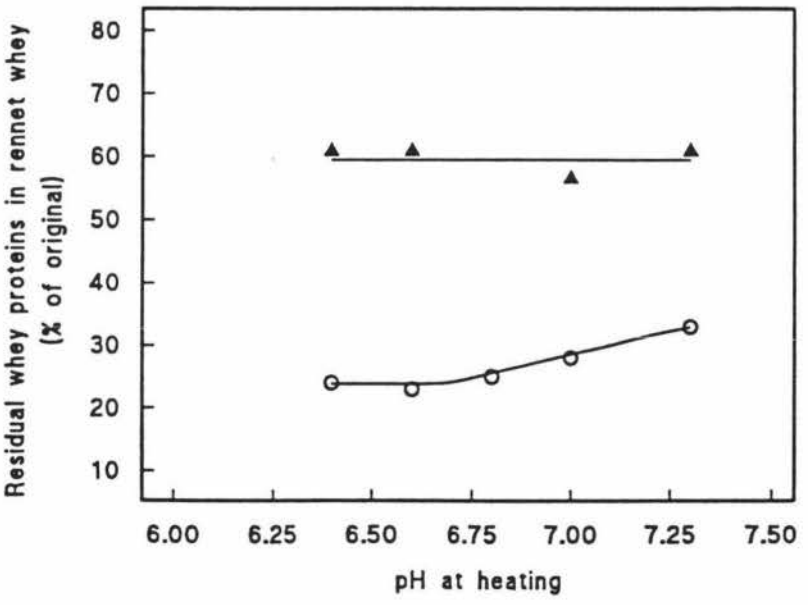

Figure 5.7: Influence of pH at heating on the amount of residual β-Lg (o) and α-La (▲) in rennet whey (estimated using quantitative SDS-PAGE). Heat treatment was at 140°C for 4 s.

5.2.2 Effect of pH at heating

Figure 5.7 shows the effect of pH at heating on the amount of whey proteins remaining soluble in the whey after renneting (at pH 6.5) of skim milk samples heated (140°C for 4 s) at different pH values.

Heating milk at pH values below pH 6.8 had little effect on the amount of β-Lg in the rennet whey. There was slightly more residual β-Lg as the pH at heating was increased to 7.0 or 7.3. No significant difference was observed in residual α-La over the same pH range.

The slight increase observed in the concentration of β-Lg remaining soluble in the rennet whey obtained from milks heated at higher pH values suggests that heat treatments at low pH values would be favoured for the incorporation of heat denatured whey proteins into cheese curds. This observation is supported by the results of Bel (1976) who claimed an optimal whey protein coagulation for inclusion in cheese if the whey proteins are heat denatured between pH 5.8 and 6.2. de Wit (1981) reported that minimal coagulation occurs when the whey proteins are heat denatured at pH >6.5.

5.3 Relationship between the state of whey proteins and their incorporation into rennet gels.

Figure 5.8a relates the amount of residual β-Lg in the rennet whey to native β-Lg resolved on Native-PAGE (Figure 5.6 versus Figure 5.1) and Figure 5.8b shows the relationship between the amount of residual β-Lg in the rennet whey and non-sedimentable β-Lg (Figure 5.6 versus Figure 5.2).

Figure 5.8a (read from right to left) shows little change in residual β-Lg in rennet whey when ≥80% of this protein was still in native form. A decrease in native β-Lg from ~80% to ~60% led to

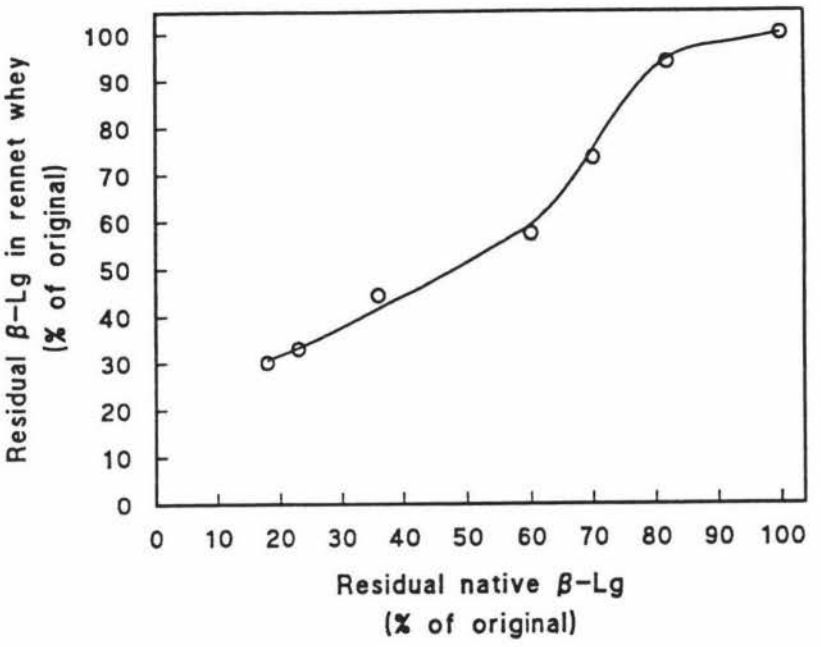

Figure 5.8a: Relationship between native β-Lg (Figure 5.1) and residual β-Lg in rennet whey (Figure 5.6)

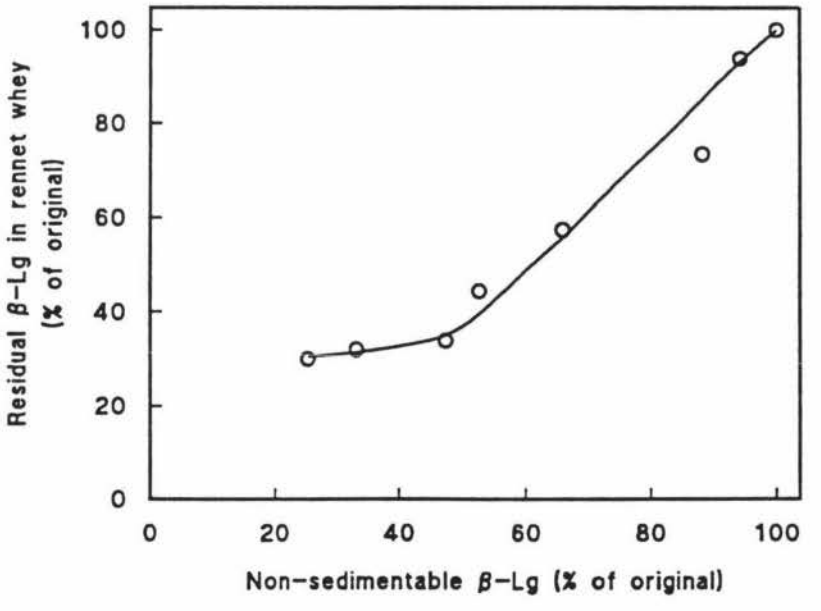

Figure 5.8b: Relationship between residual β-Lg in rennet whey (Figure 5.6) and non-sedimentable β-Lg (Figure 5.2).

a corresponding rapid decrease in residual β-Lg in the rennet whey (from ~90% to ~60%). Further loss of native β-Lg resulted in a slower decrease in residual β-Lg in rennet whey.

Figure 5.8b (read from right to left) shows that a decrease in the amount of non-sedimentable β-Lg down to ~50% of the original raw milk resulted in an almost linear decrease in the quantity of β-Lg residual in rennet whey. Further loss in non-sedimentable β-Lg led to no significant change in the amount of β-Lg residual in the rennet whey.

Overall, the interpretation of the data presented in Figures 5.8a and 5.8b suggests that up to 20% denaturation of β-Lg in milk has little effect on its incorporation into rennet gels. Between 20% and 40% β-Lg denaturation leads to rapid incorporation while at greater extents of β-Lg denaturation, the amounts incorporated increase slowly. There is a good correlation between the amounts of β-Lg associated with the casein micelles and those incorporated into rennet gels (Figure 5.8b) at levels of β-Lg association <50%. This implies that association of β-Lg with the κ-casein on the casein micelles is a pre-requisite for the incorporation of whey proteins into rennet gels. Association of more than 50% of the β-Lg with casein micelles, however, results in little further incorporation into rennet gels suggesting that only ~50% association is necessary for maximum incorporation. Association beyond this extent leads to little further incorporation.

Recently, Law *et al.* (1994) obtained good correlation between the fraction of β-Lg and α-La remaining soluble in acid filtrate (pH 4.6), and the corresponding fractions remaining in the whey obtained during production of Cheddar Cheese from pasteurised milk (72°C for 16 s) or from milks heated at 90°C for 30 s between pH 6.2 and 9.1. They concluded that the changes which occur on denaturation and cause loss of solubility at pH 4.6, such as increased hydrophobicity (Parnell *et al.* 1988; Regester *et al.* 1992),

disulphide linkage (Sawyer, 1969) and association with casein micelles (Noh and Richardson, 1989), promote the incorporation of denatured whey proteins into the curd on renneting.

5.4. Effect of heat treatment on rennet coagulation properties of milk

The conditions used for these experiments were selected from normal procedures used in cheesemaking. The results of a typical rennet coagulation process, obtained using the Bohlin (VOR) Rheometer (see Chapter 4) are shown in Figure 5.9. Figure 5.9 shows, in addition to the measured complex modulus (G*) and phase angle (δ), the calculated storage modulus (G′) and loss modulus (G"). G′ which represents the curd elasticity or firmness (Guinee *et al.* 1994) was taken as the measure of gel strength in this study.

The storage modulus (G′) started to increase about 15 min after addition of rennet indicating onset of the secondary phase of milk coagulation. A sigmoidal shaped curve of G′ *vs* time was then obtained.

The transition from sol to gel was evident from the sudden decrease of the phase angle. Milk is a viscous fluid (δ = 90°), whereas the milk gel is characterised by a phase angle of around 15°. In both these states, samples investigated could be considered homogenous, whereas there probably was an intermediate condition where viscous fluid was mixed with particles of gel.

The phase angle was practically constant during the whole build up of gel strength. The ratio of G′/G″ = tan δ gives a direct measure of the relative importance of viscous and elastic effects in the sample.

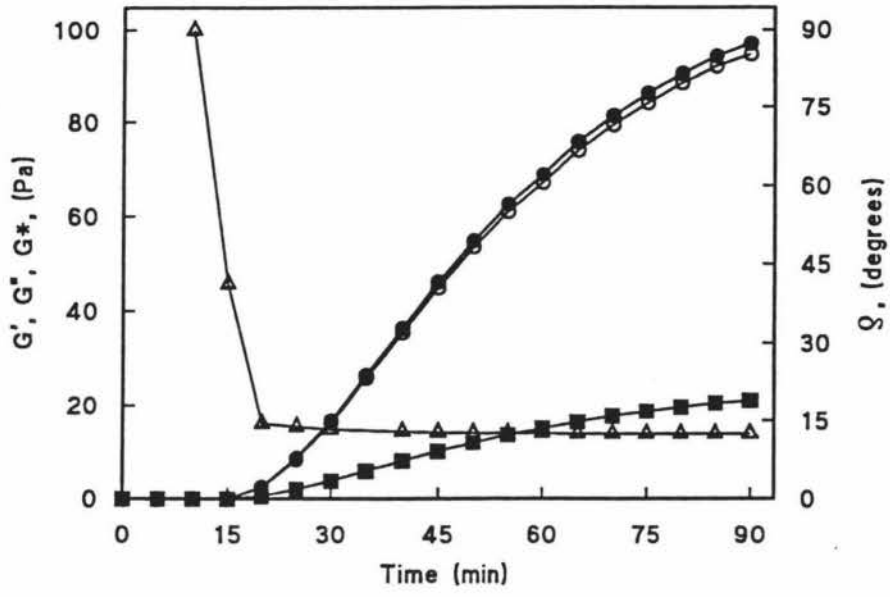

Figure 5.9: The changes of moduli and phase angle with time during the development of rennet gels. Complex modulus, G* (•); storage modulus, G′ (○); loss modulus, G″ (■); phase angle, δ (△). Zero time indicates addition of rennet.

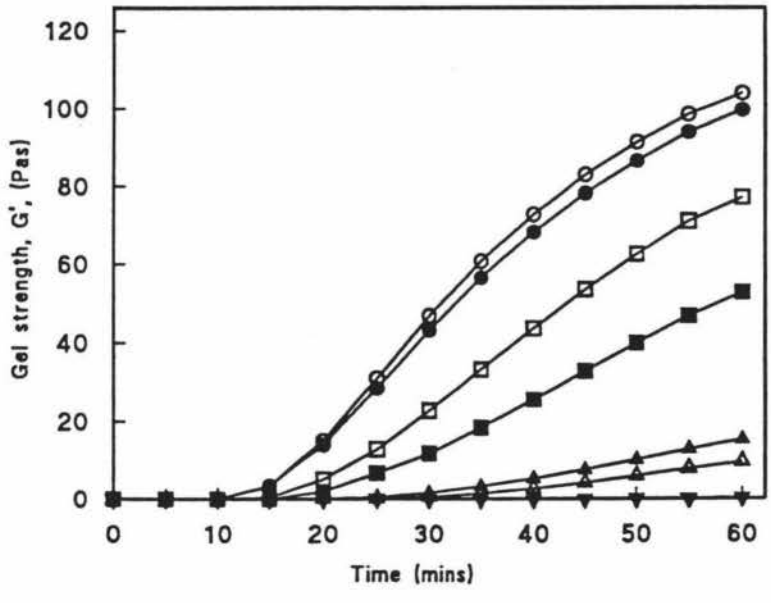

Figure 5.10: The changes in gel strength (G′) with time during rennet coagulation of heated skim milks. Unheated control skim milk (○), skim milk heated at 80 (•), 90 (□), 100 (■), 110 (▲), 120 (△), 130 (▽) or 140°C (▼) for 4 s.

The terms used in this study were obtained from the measured parameters described above and are defined as follows: the gelation time (GT) was taken as the point where there was a drop in the phase angle from 90° to ~15° which also corresponded with increase in G′. The gel strength at any time was taken as the G′ reading at that time and the rate of curd firming (K), was depicted by the initial slope of the G′ *vs* time curve (obtained using linear regression); usually up to ~40 min after GT.

5.4.1 Effect of heating temperature

Skim milk samples were heated at temperatures in the range 80 - 140°C for 4 s and tested for rennet coagulation properties as described in Chapter 4.

Figure 5.10 shows the effect of heat treatment of milk on the changes in G′ with time. G′ for the unheated control and milk heated at 80°C for 4 s began to rise at ~15 min after renneting indicating the formation of the gel network. This was followed by a rapid increase in G′ with time. The shapes of the G′ *vs* time curves changed continuously as the heat treatment became more severe. The rate of increase in G′ became progressively slower as the heating temperature increased above 90°C.

The effects of heating temperature on the various aspects of the rennet coagulation of milk (i.e. GT, K and G′) are discussed in detail below.

Gelation time (GT)

The effect of heating on the gelation time (GT) was to render the milk less susceptible to coagulation (Figure 5.11) which is consistent with the observations of previous workers (Morrisey, 1969; van Hooydonk *et al.* 1987; Singh *et al.* 1988; Dalgleish, 1990a; Lucey *et al.* 1993a).

With the temperature-time heating profiles used in these experiments, temperatures up to 100°C had relatively little effect on GT. Above 100°C, GT increased steadily with temperature up to 120°C. Between 120°C and 130°C, there was a sharp increase in GT and milk heated at 140°C failed to show a distinct transition from sol to gel in 1 h.

Rate of curd firming (K) and final gel strength (G')

Figure 5.12 shows the effect of heating temperature on K and G' (average value after 1 h) of the gels formed from heated milks. Heat treatment at 80°C for 4 s caused very little change in both K and G' but there was a progressive drop in K and G' as the heating temperature was increased from 80 to 120°C. More severe heat-treatment (130°C and 140°C) resulted in extremely low values of K and G'.

Several previous workers have also reported that the strength of rennet gels is adversely affected by heat treatment of milk (Dill and Roberts, 1959; Mauk and Demmott, 1959; Ashworth and Nebe, 1970; Singh *et al.* 1988; McMahon *et al.* 1993; Lucey *et al.* 1993a). This reduction in gel strength is presumably caused by association of denatured whey proteins with the casein micelles. The denatured whey proteins may sterically hinder the close approach and contact between renneted casein micelles, resulting in a weaker, loser network due to reduced cross-linking.

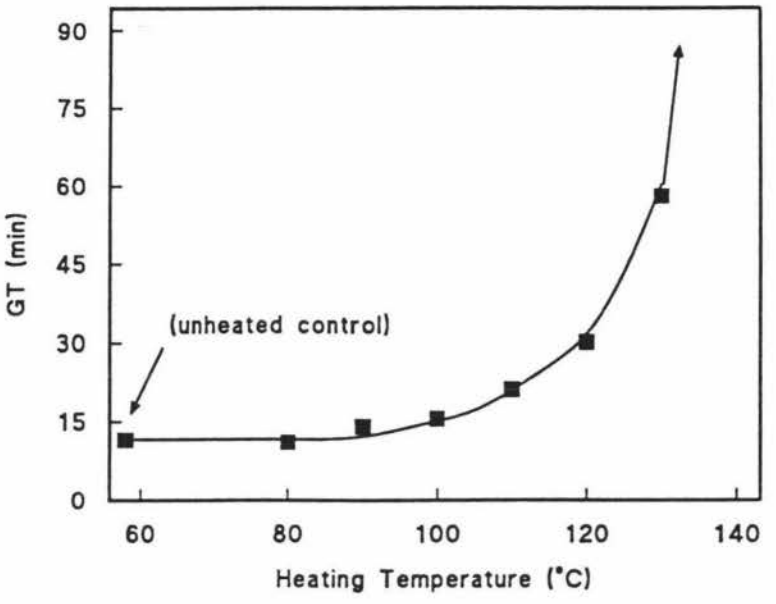

Figure 5.11: Gelation times (GT) of skim milks which have been heated for 4 s at temperatures in the range 80 - 140°C.

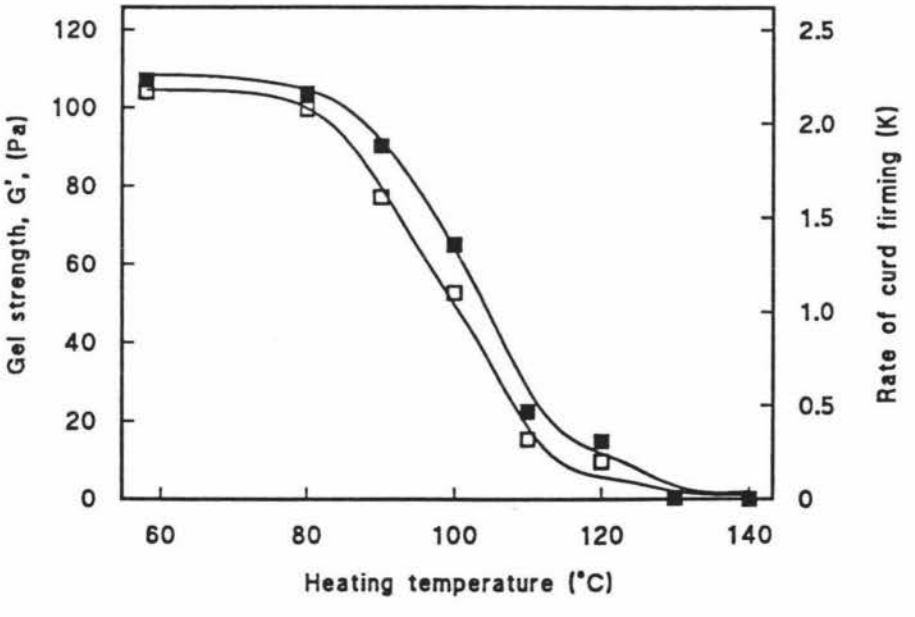

Figure 5.12: Gel strengths, G', (□) and rates of curd firming, K, (■) for skim milks which have been heated for 4 s at temperatures in the range 80 - 140°C.

5.4.2 Effect of pH at heating

Milk samples were adjusted to pH values in the range 6.4 to 7.3 and heated at 140°C for 4 s. Samples were then acidified from their respective heating pH to pH 6.5 before renneting.

Figure 5.13 shows the changes in G′ as a function of renneting time. The heated milks all showed atypical G′ *vs* time curves due to severe retardation of the secondary phase of renneting and all G′ (average after 2 h) values were <5 Pa. The three parameters (GT, K and G′) are discussed in more detail below.

Gelation time

Difficulties were encountered in determination of GT in these samples since a distinct permanent drop in the phase angle from 90° was not observed immediately (results not shown). Instead, during the early stages of coagulation, there was a period during which the phase angle fluctuated between 90° and ~15°. Therefore, the GT was taken as the average time from when there was the first sign of a drop and when this became permanent.

The pH at heating had an effect on GT, particularly in the pH range 6.6 to 7.3 where the GT increased with increase in pH from 6.6 to 7.0 but decreased at higher pH values (Figure 5.14). When compared to the unheated control sample, however, all the heated samples had much longer GT.

Singh *et al.* (1988) heated milk to various temperatures (65 - 95°C) over the pH range 6.5 - 7.0. Upon renneting at pH 6.5 (30°C), they observed that the RCT of samples heated at pH 6.5 increased to a lesser extent (from 3 to 8 min) than for those heated at pH 7.0 over the same temperature range which increased from about 3 to 35 min. The results presented here show a similar trend in the pH range 6.4 - 7.0. At pH 7.3,

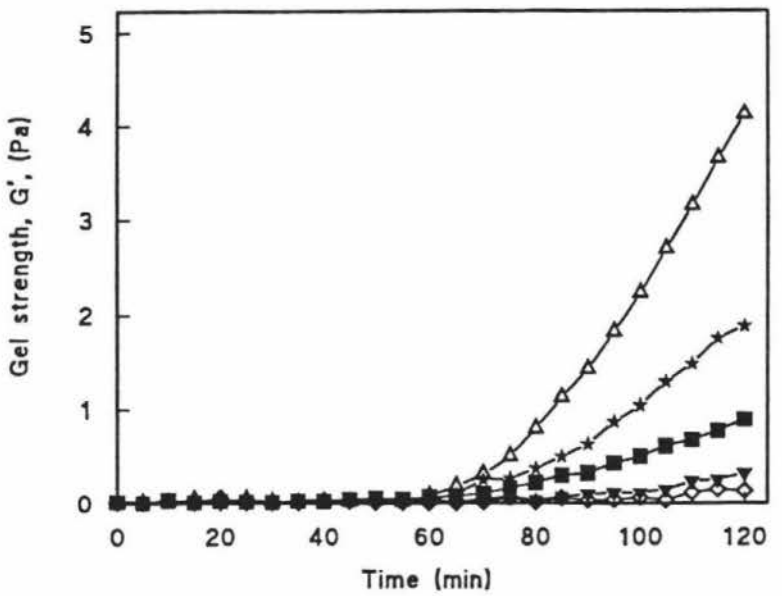

Figure 5.13: Influence of pH at heating on the changes in G′ with time. Skim milk samples were adjusted to pH 6.4 (■), 6.6 (▵), 6.8 (▾), 7.0 (◊) or 7.3 (★) and heated at 140°C for 4 s. The pH of all samples was readjusted to 6.5 before renneting.

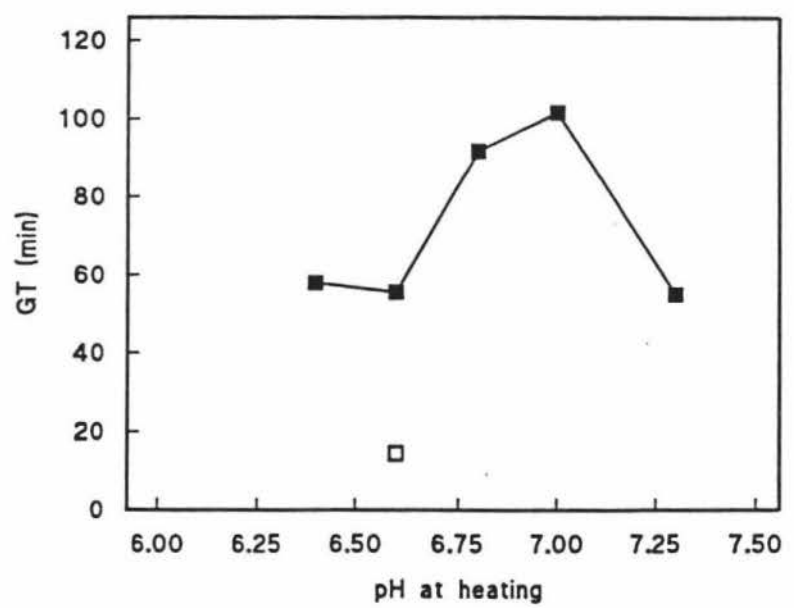

Figure 5.14: Gelation times of skim milks which have been adjusted to pH values in the range 6.4 - 7.3 and heated at 140°C for 4 s. Heated samples (■), unheated control (□). The pH of all samples was readjusted to 6.5 before renneting.

however, the GT was shortened again to values similar to those below pH 6.8.

Rate of curd firming (K) and final gel strength (G')

Figure 5.15 shows that the K and G′ of heated milks were not significantly influenced by pH at heating except that milk heated at pH 6.6 had slightly higher K and G′ values. All samples formed very weak gels (G′ < 5Pa) and had correspondingly low K values.

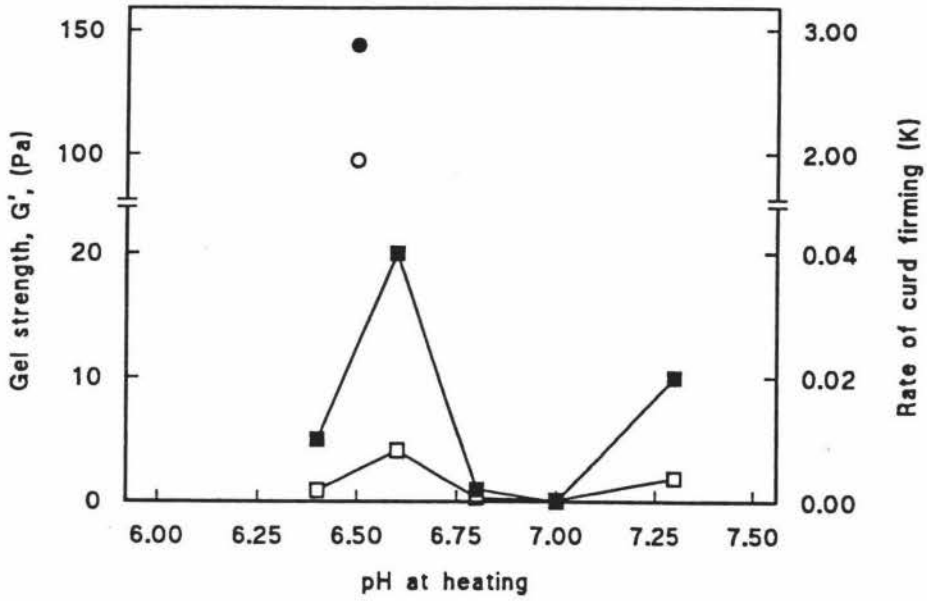

Figure 5.15: Gel strength, G′, (□) and rate of curd firming, K, (■) for skim milks which have been adjusted to pH values in the range 6.4 - 7.3 and heated at 140°C for 4 s. G′ for unheated control (o), K for unheated control (•). Renneting was carried out for all samples at pH 6.5.

The present study showed that considerably less β-Lg was associated with casein micelles on heating milk at elevated pH which could be due to dissociation of the β-Lg/κ-casein complex from the casein micelles into the serum upon (see Figure 5.4). This should thus reduce the zeta potential

(through reduction of the surface charge of the micelles as a result of loss of κ-casein) of the casein micelles. It was thus expected that this coupled with the presence of lower amounts of denatured whey protein on the micelle surface (due to dissociation of the β-Lg/κ-casein complex) at high pH should promote aggregation of renneted micelles and thus improve the rennet coagulation properties of heated milks.

The results above indicate, however, that no such improvement was possible. This agrees with van Hooydonk *et al.* (1987) who adjusted the pH of milk to 7.5 before heating (90°C for 10 min) followed by acidification to pH 6.7 before renneting. They further reported that the aggregation of the rennet-converted micelles in an unheated sample that had been adjusted to pH 7.5 and then re-neutralised to pH 6.7 before renneting was also substantially retarded. They concluded that this was because stable Ca/P complexes (similar to those formed on heating milk) which lack the association properties of micellar calcium phosphate (MCP) may be formed at high pH even without any heat treatment and may offset any positive effects that may have been gained through heating at elevated pH.

Singh and Fox (1986) also found no improvement in the visual rennet coagulation time (RCT) after heating milk at pH 7.3 and acidifying to pH 6.7 before renneting. They suggested that readjustment of pH to 6.7 before renneting may have caused reassociation of κ-casein and whey protein with the micelles and thus reintroduced adverse renneting properties.

To verify this latter suggestion, milk was heated (140°C for 4 s) at pH values in the range 6.4 - 7.3. Sub-samples were then adjusted to pH 6.5, and ultracentrifuged (100,000*g* for 1h). Supernatants from all samples were then analysed for β-Lg and κ-casein using SDS-PAGE. The results showed that the amounts of non-sedimentable β-Lg and κ-casein after heating milk at pH 7.0 and 7.3 were markedly reduced upon acidification to pH 6.5 (Figure 5.16). This, indeed, shows that dissociation of the β-

Lg/κ-casein complex at high pH values is at least partially reversible upon acidification as explained by Singh and Fox (1986). Acidification to pH 6.5 after heating at pH values >6.8 thus led to re-association of the β-Lg/κ-casein complex onto the surface of the casein micelles and so possibly re-introduced adverse renneting properties.

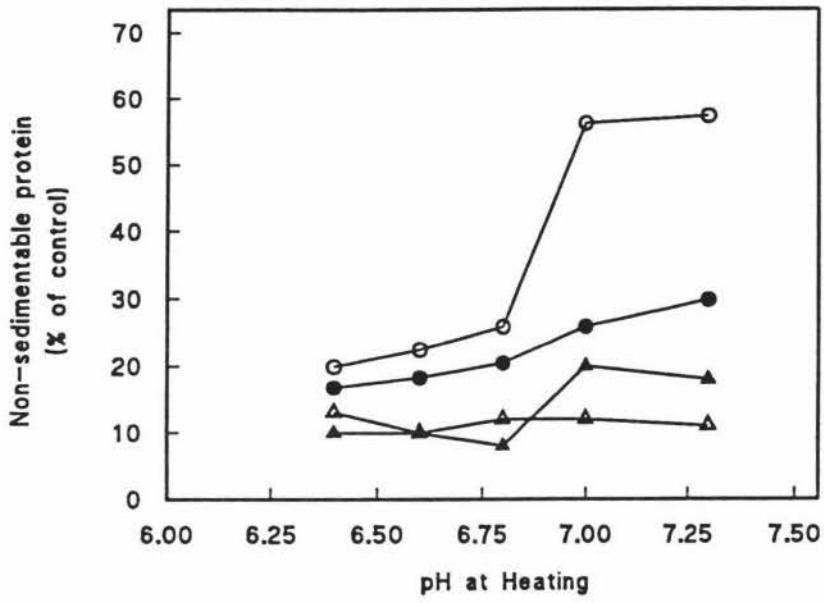

Figure 5.16: The pH-dependent reversibility of the amounts of non-sedimentable β-Lg and κ-casein. Amounts of non-sedimentable (100,000*g* for 60 min) β-Lg (○) and κ-casein (▲) at the heating pH. Amounts of non-sedimentable β-Lg (●) and κ-casein (△) after readjustement to pH 6.5.

5.5. Relationship between the state of whey proteins and rennet coagulation properties

Combination of the results obtained for residual native β-Lg (Figure 5.1) and non-sedimentable β-Lg (Figure 5.2) discussed earlier and those for the effect of heat treatment of milk on GT and G′ discussed above reveals an interesting relationship between these processes.

5.5.1 Relationship between the state of whey proteins and GT

Figure 5.17 relates the amounts of residual native β-Lg or non-sedimentable β-Lg to GT (Figures 5.1 and 5.2 versus Figure 5.11). It is clear that GT was significantly lengthened as the amounts of residual native β-Lg or non-sedimentable β-Lg decreased. Exponential curves were obtained for both the effects of residual native β-Lg and of non-sedimentable β-Lg on GT. These curves were, however, displaced from each other and increases in GT were observed at much lower levels of β-Lg association than denaturation.

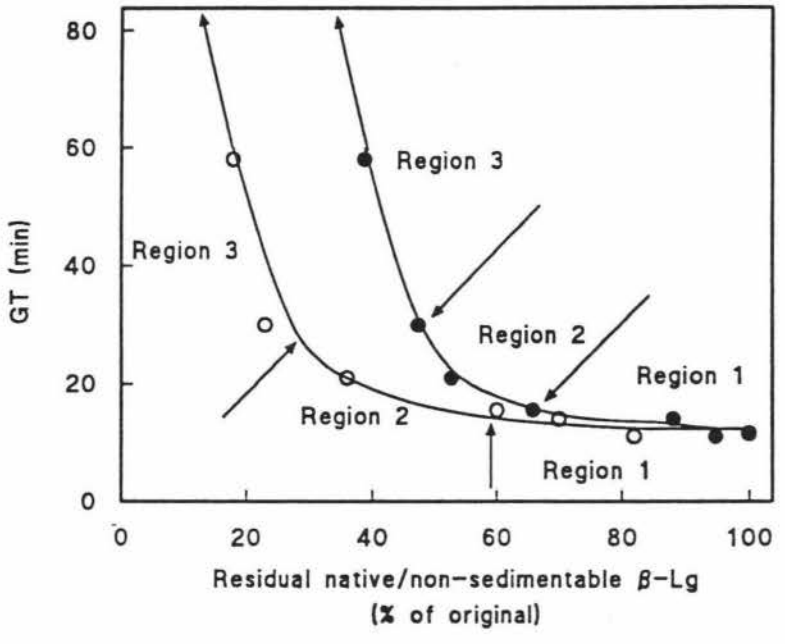

Figure 5.17: Relationship between residual native β-Lg (o) (from Figure 5.1), or non-sedimentable β-Lg (•) (from Figure 5.2) and gelation time (from Figure 5.11).

Very little change was observed in GT as the quantity of residual native β-Lg decreased to ~60% of the original amount in raw (unheated) milk. GT was gradually lengthened as more native β-Lg was lost (from ~60% to ~35%). Finally, further loss of native β-Lg (<35% residual) led to a very sharp increase in GT. Heated milk with <20% residual native β-Lg had GT >60 min.

GT increased very slowly as the amount of non-sedimentable β-Lg decreased to ~65% of the original amount in raw milk. The slope of the curve steadily increased as the concentration of non-sedimentable β-Lg decreased (from ~65% to ~50%). Finally there was a sharp increase in GT when the concentration of non-sedimentable β-Lg decreased to <50% of the original. Milk with less than ~40% non-sedimentable β-Lg had GT >60 min.

The results obtained for the relationship between GT and denatured β-Lg are in good agreement with those obtained by Dalgleish (1990a) for the relationship between RCT and denatured β-Lg.

These results show that the effects of β-Lg denaturation and association with casein micelles on GT may be divided into 3 distinct regions. Region 1 consists of the period during which there was <40% denaturation or <35% association of β-Lg with casein micelles. In this region there was very little change in GT. This suggests that relatively mild heat treatments resulting in low levels of β-Lg denaturation or association have little effect on the aggregation of renneted micelles during the early stages of the secondary phase of rennet coagulation of milk.

More severe heating conditions (resulting in β-Lg denaturation between 40% and 65% or association between 35% and 50%) gave rise to region 2. In this region, denatured β-Lg molecules began to attach to the casein micelles in high enough concentrations as to interfere with the aggregation of renneted micelles resulting in increased GT.

Finally, in region 3, GT was extensively lengthened despite little change in β-Lg denaturation (from ~65 to ~80%) or association (from ~50% to ~60%). The interference of the denatured whey protein with the aggregation of renneted micelles could not have become much worse in this region due to the relatively minor changes in denatured and associated β-

Lg. The excessive lengthening of GT observed in this region can thus be attributed to the more extreme heating conditions causing chemical damage to the κ-casein molecules and possibly decreasing the rate and extent of hydrolysis during the primary phase.

5.5.2. Relationship between the state of whey proteins and G′

A sigmoidal relationship was observed between G′ and residual native β-Lg (Figure 5.18). Loss of native β-Lg to ~80% of the original (i.e. 20% denaturation) had little effect on G′. There was a sharp drop in G′ as the amount of residual native β-Lg decreased from ~80% to ~35%. Further decrease led to a more gradual drop in G′ and milks containing <20% native β-Lg formed gels of negligible strength.

As the amount of non-sedimentable β-Lg decreased to ~50% of the original, there was an almost linear decrease in G′ (Figure 5.18). Milks containing <50% non-sedimentable β-Lg formed gels of negligible strength.

The results presented above indicate that the G′ of rennet gels formed from heated milks is more sensitive to the presence of denatured and associated β-Lg than the GT. This is clearly demonstrated in Figure 5.19 which was obtained through superimposing Figure 5.17 on Figure 5.18. Figure 5.19 shows that the GT did not begin to lengthen until at least 40% denaturation of β-Lg. The G′, however, started to drop at a lower extent of β-Lg denaturation (~20%). When examined as a function of β-Lg association, it is evident that GT only started to be significantly affected at extents of association of ~35% whereas G′ started to drop significantly at ~10% association. These observations suggest that the secondary phase of rennet coagulation (i.e. development of gel structure and gel strength) is more sensitive to pre-heat treatment of the milk than the rennet clotting time.

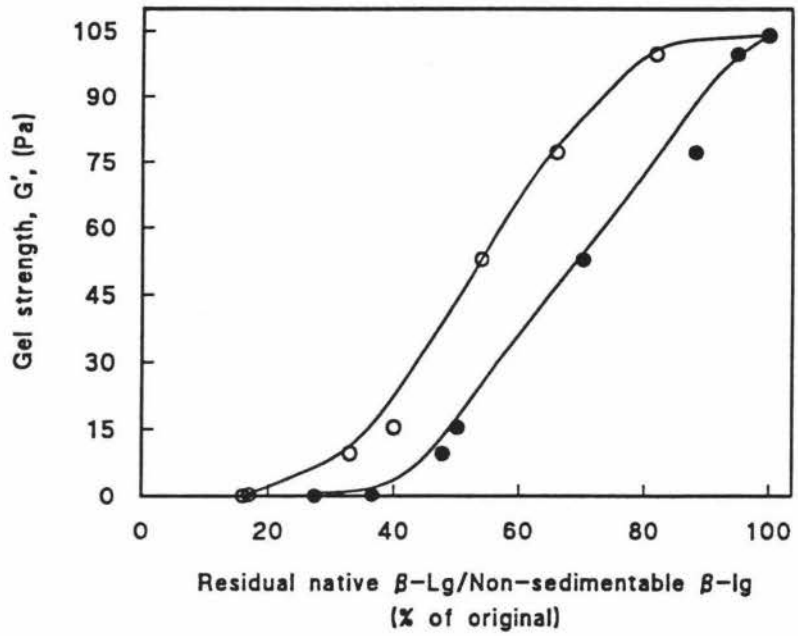

Figure 5.18: Relationship between the amounts of residual native β-Lg (o) (from Figure 5.1) or non-sedimentable β-Lg (•) (from Figure 5.2) and gel strength (G') (from Figure 5.12).

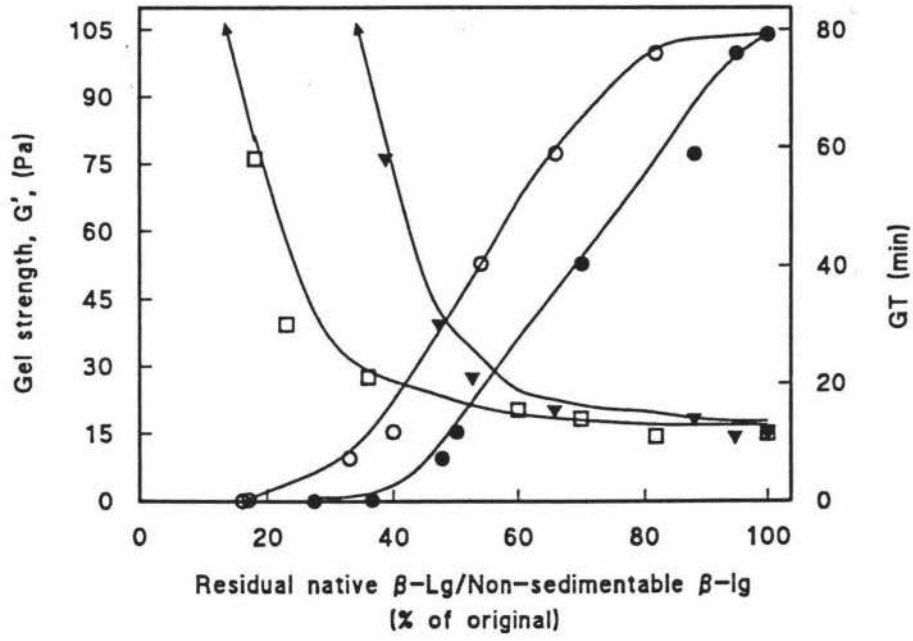

Figure 5.19: Relationship between residual native β-Lg (o) or non-sedimentable β-Lg (•) and G' or GT.

Development of gel structure is due to the formation of a limited number of reactive sites at the micelle surface in the course of renneting. In raw milk, the reactive sites of adjacent micelles fuse together and eventually form a 'bridge' which is kept together by several bonds which may be of different and changing natures. For instance, initial bonding may be in the form inter-particle Van der Waals attractions, these may then be followed by simple salt bridges and hydrophobic bonding (Walstra and van Vliet, 1986). The increase in gel strength (G′) with time (e.g. Figure 5.9) could be attributed to increase in the proportion of gelled material (i.e. increase in network density), increase in the number of cross-links and regularity of the network and also an increase in the number of bridges as well as in the number and strength of bonds per bridge (Johnston, 1984; Walstra and van Vliet, 1986).

In heated milk, association of whey proteins onto the surfaces of casein micelles through formation of a β-Lg/κ-casein complex may limit or decrease the number of reactive sites on renneted micelles. This in turn would lead to a decrease in the chances of fusion between the reactive sites of adjacent renneted micelles, resulting in formation of fewer bridges with less and weaker bonds. Alternatively, association of β-Lg with the casein micelles may simply hinder the close approach of the reactive sites even if there are sufficient sites for formation of the bridges. These effects worsen with the severity of heat treatment of the milk which may account for the delayed gel appearance (i.e. lengthened GT), slow gel development (i.e. decreased K) and weakened gels (i.e. low G′) (see Figures 5.17 to 5.19).

The G′ of the rennet gels is probably more important than the GT in practical cheesemaking since it gives a better estimation of the gel strength at cutting and also of the firmness of the cheese. Bohlin *et al.* (1984) suggested that a G′ of ~30Pa is desired at the time of cutting the curd during cheese manufacture which is normally about half an hour after rennet addition. The results for G′ shown in Figure 5.18 (which were

recorded 1 h after renneting) imply that heat treatments resulting in ~30% association or ~45% denaturation of β-Lg is the cut-off point beyond which the strengths of the rennet gels formed from heated milks are lowered to such an extent that cheese manufacture from this milk becomes impracticable.

5.6 Conclusions

The following general conclusions may be drawn from the results discussed in this Chapter:

(i) Upon heating milk, the denaturation of the β-Lg and α-La is a much faster process that their association with the casein micelles.

(ii) The β-Lg/κ-casein complex shows pH-dependent dissociation and heating of milk at elevated pH results in its dissociation from the micelles into the serum.

(iii) There is good correlation between the amount of β-Lg associated with the casein micelles and that incorporated into rennet gels but only up to 50% association.

(iv) The rennet coagulation properties of skim milk are adversely affected by heat treatment resulting in longer gelation times and lower gel strengths.

(v) Up to ~40% denaturation of β-Lg or (~35% association of β-Lg with the casein micelles) has little influence on gelation times. Higher levels of denaturation and association result in significant increases in gelation time.

(vi) Up to ~20% denaturation of β-Lg has little influence on the gel strength but higher levels of denaturation result in a rapid decrease. There is an almost linear decrease in gel strength with increase in the extent of β-Lg association with casein micelles up to ~50% association. Milks containing >50% associated β-Lg form gels of neglible strength.

(vii) Heat treatment of milk at elevated pH does not significantly improve its rennet coagulation properties over those of milk heated at the normal pH range.

(viii) The development of the rennet gels and the final gel strengths obtained are more sensitive to the presence of denatured and /or associated whey proteins than the gelation time.

CHAPTER 6

RENNET COAGULATION PROPERTIES OF HEATED MILKS: EFFECTS OF pH ADJUSTMENT AND $CaCl_2$ ADDITION

Acidification of heated milk to low pH values (≤5.5) followed by re-neutralisation to the natural pH range, often termed as pH cycling, (van Hooydonk *et al.* 1987; Singh *et al.* 1988; Banks, 1988; Lucey *et al.* 1993a), heating at elevated pH (Singh and Fox 1986; van Hooydonk *et al.* 1987), heating at elevated pH combined with pH cycling (Imafidon and Farkye, 1993), and $CaCl_2$ addition (Bohlin *et al.* 1984; van Hooydonk *et al.* 1987; Singh *et al.* 1988; Lucey *et al.* 1993a) have been used previously in attempts to improve the rennet coagulation properties of heated milks. In this study, attempts were made to improve the gel forming characteristics of heated milks using the above techniques as well as combinations of them.

6.1 Effect of pH cycling on the rennet coagulation properties of heated milks

6.1.1 Effect of heating temperature

Skim milk samples were heated at temperatures in the range 80 - 140°C. An unheated sample from the same batch of bulk milk was used as a control. Each milk sample was sub-divided into two sub-samples of which one was directly acidified to pH 6.5 and the other acidified to pH 5.5, held for 2 h, and subsequently neutralised to pH 6.5 (pH cycled). This procedure is outlined in the diagram below:

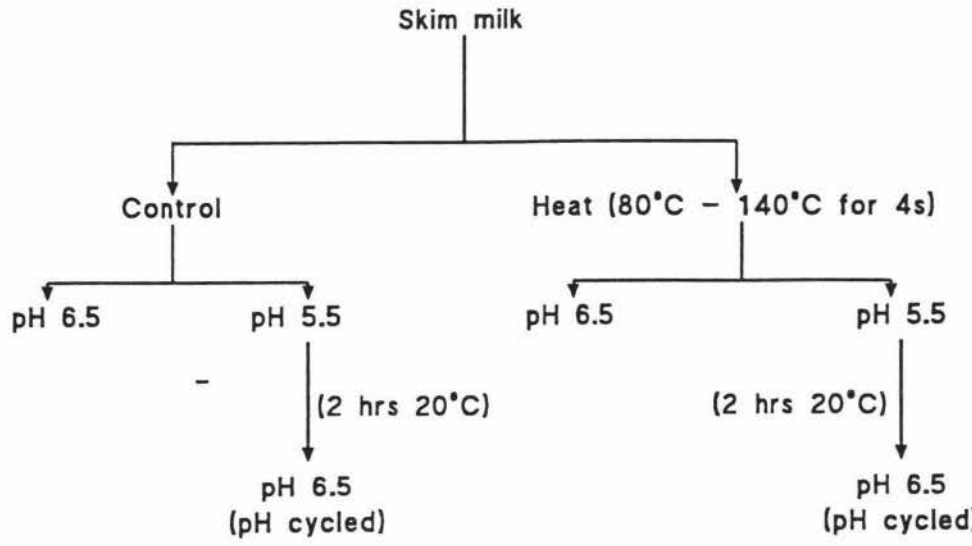

Figure 6.1: Experimental procedure for determining the effects of acidification and subsequent reneutralisation (i.e. pH cycling) on the rennet coagulation properties of heated milks.

Gelation time (GT)

The effect of pH cycling on the GT of heated milks is shown in Figure 6.2. GT decreased in all samples upon pH cycling; the decrease in GT was greatest when the milks were heated either at 100°C or at 120°C. At 140°C, there was only ~10% reduction in GT.

Singh *et al.* (1988) heated milk at 90°C for 10 min before adjusting the pH from 6.6 down to values in the range 5.1 - 6.3, holding at the low pH for 2 h at 20°C and then readjusting to pH 6.6 before measurement of RCT at 30°C. They reported marked reductions in the RCT of such pH cycled milk. van Hooydonk *et al.* (1987), who used more severe heat treatment (5 s at 140°C, direct UHT) observed reductions in the GT of about 15% and 14% upon acidification to pH 6.0 and 5.5 respectively (re-neutralisation was carried out after 24 h). More recently, Lucey *et al.* (1993a) reported reductions in RCT for milks heated at temperatures up to 100°C for 10 min. More severely heated milk (120°C for 10 min) did not coagulate even after acidification to low pH values and re-neutralisation to pH 6.6.

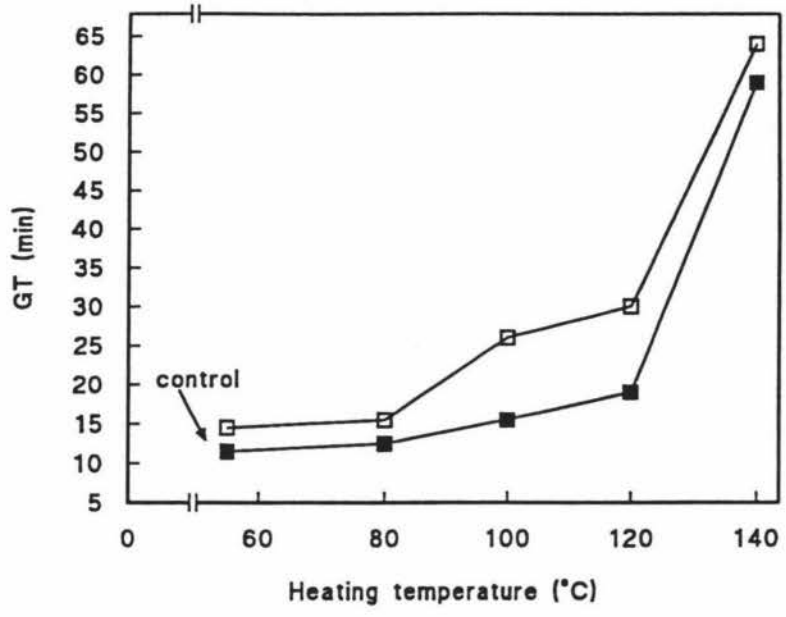

Figure 6.2: Gelation times (GT) of skim milks heated at temperatures in the range 80 - 140°C for 4 s (□). Heated skim milks adjusted to pH 5.5, left for 2 h at 20°C, and readjusted to pH 6.5 (■) before renneting. All samples were renneted at pH 6.5.

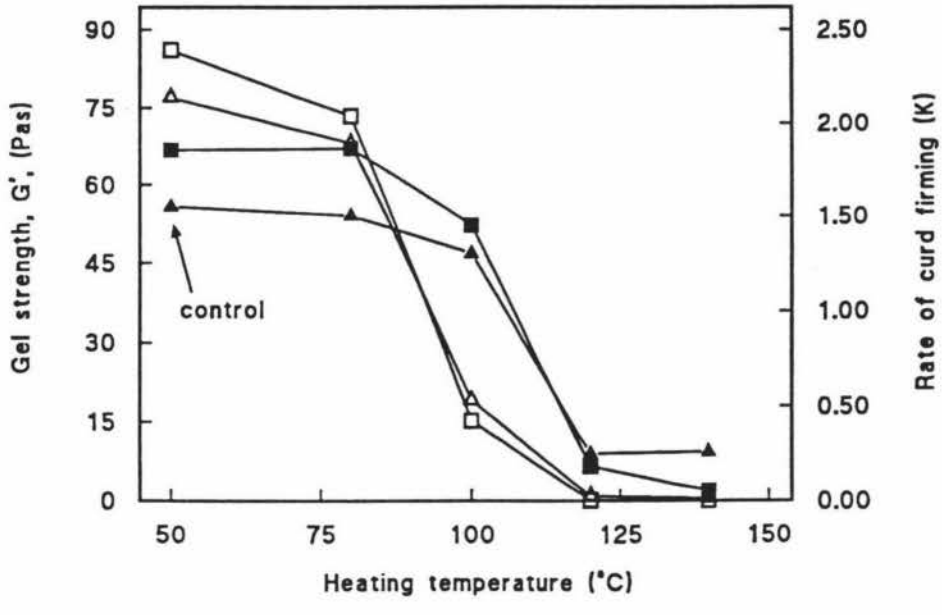

Figure 6.3: Gel strengths, G′, (□) and rates of curd firming, K, (△) of skim milks heated at temperatures in the range 80 - 140°C for 4 s. G′ (■) and K (▲) of heated skim milks adjusted to pH 5.5, left for 2 h at 20°C, and readjusted to pH 6.5 before renneting.

Rate of curd firming (K) and final gel strength (G')

pH cycling had an effect on the strength of the rennet gels formed from heated milks (Figure 6.3). In the unheated (control) milk, pH cycling had an adverse effect on K and G' and this persisted to a smaller extent in the sample heated at 80°C. The most significant increase in K and G' was observed in the sample heated at 100°C; samples that had received more severe heat treatments prior to renneting also formed firmer gels upon renneting after pH cycling, although the improvements were not as pronounced as that observed at 100°C.

Singh *et al.* (1988) reported that the rennet gel formed from milk heated at 90°C for 10 min, acidified to pH 5.5 and re-adjusted to pH 6.6, was slightly more firm than that from the original, unheated milk. Lucey *et al.* (1993a), however, found that acidification of heated milk (100°C for 10 min) to pH values of ≤5.5, before re-neutralization to pH 6.7, resulted in greatly improved RCT but gel firmness was not completely restored to that of raw milk.

Mechanism of pH cycling effect.

The concept of pH cycling as a means of improving the rennet coagulation properties of heated milks was initiated by experiments performed by Banks and Muir (1985). These showed that sterilised starter milk, which was acidified by lactic acid and subsequently neutralised by addition to the cheese milk, fully incorporated into the rennet gel. They suggested that this was due to the disruption of micelles at low pH, which would make the hidden κ-casein liable to conversion and cause the casein micelles to participate in the gel formation process. It has since been shown, however, that the pH cycling procedure does not result in the release of any additional casein macropetide (CMP) (van Hooydonk *et al.* 1987) or 12% TCA soluble N-acetylneuramic acid (NANA) (Singh *et al.* 1988) during the primary enzymic phase of the coagulation of heated milks. The improvement of rennetability of heated milks cannot, therefore, be

explained by the mechanism suggested by Banks and Muir (1985). Rather, van Hooydonk *et al.* (1987) suggested that acidification to pH 5.5 solubilises a substantial amount of both the original colloidal calcium phosphate (CCP) and the heat-induced Ca/P complexes. The subsequent neutralisation in the cold probably leads to the reformation of Ca/P complexes with composition and properties more like the original CCP and this may be the most important factor responsible for the improved rennet coagulation properties of heated and pH cycled milks. Singh *et al.* (1988) reported that pH cycling affected the rennet coagulability of heated milk by increasing the $[Ca^{2+}]$, (an effect similar to that obtained by small additions of $CaCl_2$), and thus promoting aggregation of renneted micelles.

Overall, it may be concluded that pH cycling partly restores the rennet coagulation properties of heated milks. The extent to which these are restored is very much dependent on the severity of the heat treatment employed. The results of this study suggest that the GT is more sensitive to pH cycling than the G′. The coagulation properties of milks heated at temperatures of about 100°C appear to be mostly restored by pH cycling but those of more severely heated milks (≥120°C) are less affected.

The failure of pH cycling to improve the rennet coagulation properties of severely heated milks may be due to increased association of β-Lg with casein micelles. This may correspond to region 3 (see section 5.5.1) in which a marked increase in GT was observed despite little change in denatured and/or associated β-Lg. Therefore, there may be a critical extent of β-Lg association with κ-casein beyond which rennet coagulation is seriously impaired and pH cycling is unable to restore favourable gel forming properties in severely heated milks.

Heat treatment of milk causes 'precipitation' of calcium phosphate and after severe heat treatment, not all the heat-precipitated calcium phosphate is solubilised on acidification (Lucey *et al.* 1993b; Singh, 1994).

It is suggested, therefore, (based on the possible mechanisms by which pH cycling improves the renneting properties of heated milks as suggested by van Hooydonk *et al.* 1987 and by Singh *et al.* 1988) that there is less reformation of calcium phosphate similar in form and structure to native CCP and/or a smaller increase in $[Ca^{2+}]$ upon re-neutralisation of previously acidified severely heated milk. This may account for the lesser improvement in the renneting properties of severely heated milks compared to that in milk that had received milder heat treatments as observed in this study.

A number of other physicochemical changes occur in casein micelles at severe heating temperatures which may influence their renneting behaviour. These include changes in the amino acid residues of the caseins such as removal of phosphate from phosphoserine (Belec and Jenness, 1962), formation of lysinoalanine (Manson and Carolan, 1980), and reaction of ε-amino groups of lysine with carbonyl groups of lactose as the first step in the formation of a series of Maillard products (Burton, 1984).

6.1.2 Effect of pH at heating

Skim milk samples were adjusted to pH values in the range 6.4 - 7.3 and heated at 140°C for 4 s. After heat treatment, each sample was subdivided into 2 sub-samples, one of which was pH cycled and the other directly acidified to pH 6.5. An unheated sample was used as a control.

Gelation time

GT of all samples decreased upon pH cycling of milks heated at different pH values (Figure 6.4). However, the GT of the unheated control samples could not be matched as a result of pH cycling of the heated milks.

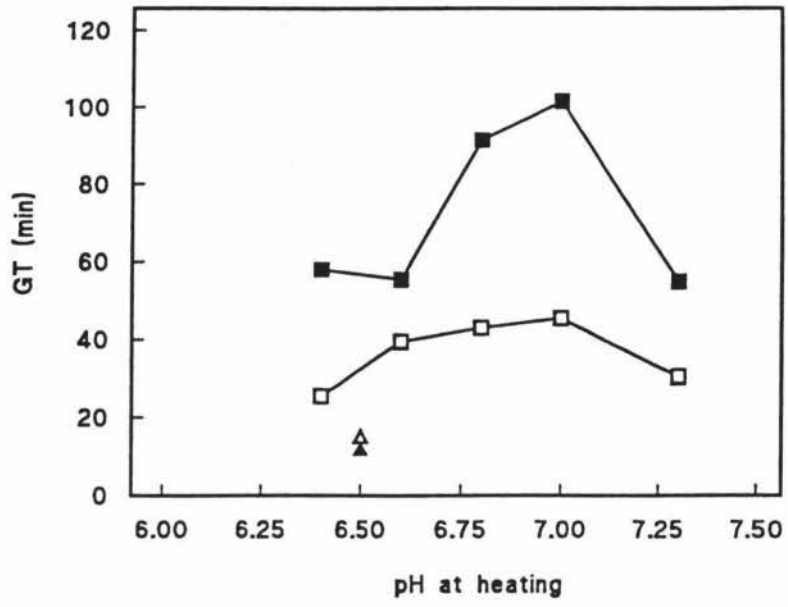

Figure 6.4: Gelation times (GT) of skim milks heated at 140°C for 4 s at pH values in the range 6.4 - 7.3 (■). Skim milks heated at 140°C for 4 s at pH values in the range 6.4 - 7.3, acidified to pH 5.5, left for 2 h at 20°C, and readjusted to pH 6.5. Raw skim milk (△), raw skim milk acidified to pH 5.5, left for 2 h at 20°C, and readjusted to pH 6.5 (▲). All samples were renneted at pH 6.5.

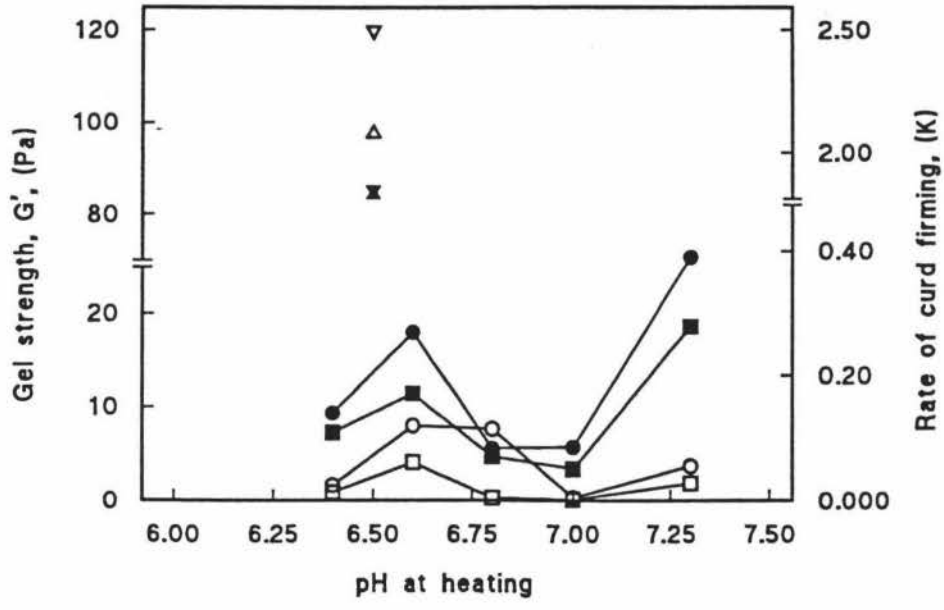

Figure 6.5: Gel strengths, G', (□) and rates of curd firming, K, (○) of skim milks heated at 140°C for 4 s at pH values in the range 6.4 - 7.3. G' (■) and K (•) of milks heated at different pH values, acidified to pH 5.5, and reneutralised to pH 6.5. G' (△) and K (▲) for raw skim milk. G' (▽) and K (▼) for raw milks which have been acidified to pH 5.5 and neutralised to pH 6.5.

Rate of curd firming (K) and gel strength (G')

Both K and G' increased for all samples after pH cycling (Figure 6.5). The greatest improvement was observed for the sample heated at pH 7.3. None of the heated samples matched K and G' values of unheated control samples.

The improvements obtained in GT, K and G' in this study were not as significant as those recorded by Imafidon and Farkye (1993). They reported that milk heated (91°C for 16 s) at pH 7.5 and directly acidified to pH 6.4 before setting had a mean RCT of ~9.41 min. After acidification to pH 5.5 and overnight storage at 4°C before re-neutralisation to pH 6.4 (pH cycling) RCT decreased by ~50%. They also reported similar changes in the RCT of more severely heated milks (91°C for 60 s) and recorded faster curd-firming rates and slightly firmer gels in samples pH cycled to pH 6.4 than in those directly acidified to the same pH. However, when milk was pH cycled to its original pH of 6.6 before setting, the curds formed were very weak. More severe heat treatments were used in the current study which may have extensively altered the properties of the milk system such that the adverse effects of heat treatment of milk on coagulation properties could not be reversed upon heating at elevated pH and pH cycling.

In summary, pH cycling slightly improved the rennet coagulation properties of milks heated at different pH values. The sample heated at pH 7.3 and pH cycled had the shortest GT and highest G' values of all the heat treated milks. These, however, were still far short of those of an unheated control, and the rennet coagulation properties of severely heated milks were not satisfactorily restored as a result of increasing the pH before heating followed by an acidification and re-neutralisation step.

6.2 Effect of $CaCl_2$ addition and pH cycling

Calcium chloride was added at two levels (1mM or 2mM) to both unheated

into 2 sub-samples, one of which was pH cycled before $CaCl_2$ addition and the other one not as shown in the diagram below.

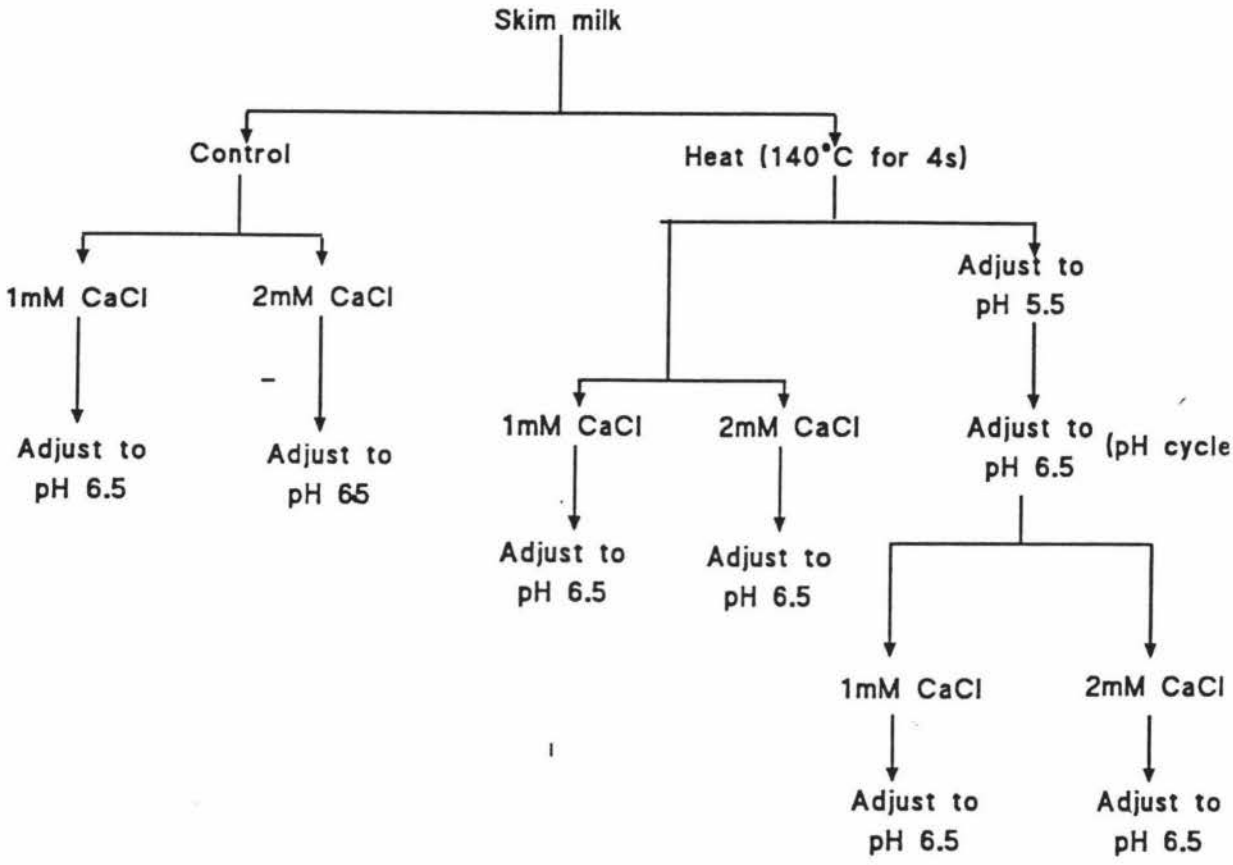

Figure 6.6: Experimental procedure for the determination of the effect of acidification and reneutralisation (pH cycling), addition of $CaCl_2$ or combined effect of pH cycling and $CaCl_2$ addition on rennet coagulation properties of heated milks.

Figure 6.7 shows the change in G′ with time (during renneting) at different levels of $CaCl_2$ addition. Generally, the rate of G′ development increased as the concentration of added $CaCl_2$ was increased in both the unheated and heated milks.

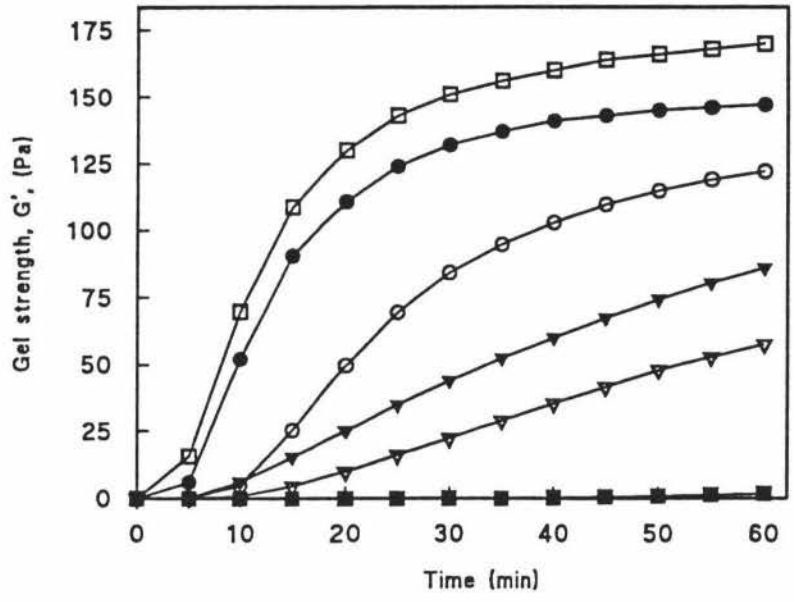

Figure 6.7: Influence of $CaCl_2$ addition on the changes in G′ with time during renneting of heated (140°C for 4 s) and unheated milks. Unheated milk with no $CaCl_2$ added (○), 1mM $CaCl_2$ added (•), or 2mM $CaCl_2$ added (□). Heated milk with no $CaCl_2$ added (■), 1mM $CaCl_2$ added (▽), or 2mM $CaCl_2$ added (▼).

The table below summarises the effect of $CaCl_2$ addition (and $CaCl_2$ addition combined with pH cycling) on the GT, K, and G′ of heated (140°C for 4 s) milk.

Table 6.1

Effect of $CaCl_2$ addition and pH cycling on the rennet coagulation properties of heated milk.

Sample	pH cycle	[$CaCl_2$]	GT (min)	(K)	G′ (Pa)
Control	-	0.00mM	7.0	3.47	122
Control	-	1.00mM	3.5	5.88	147
Control	-	2.00mM	2.0	7.63	170
140°C (4s)	-	0.00mM	62.5	0.03	2
140°C (4s)	-	1.00mM	8.0	1.21	58
140°C (4s)	-	2.00mM	4.5	1.62	86
140°C (4s)	5.5 - 6.5	0.00mM	54.5	0.17	10
140°C (4s)	5.5 - 6.5	1.00mM	6.5	1.17	57
140°C (4s)	5.5 - 6.5	2.00mM	3.5	1.43	79

Gelation time (GT)

Table 6.1 shows that the GT of the unheated control decreased as the concentration of added $CaCl_2$ was increased. For the heated milk, there was a marked drop in GT upon addition of 1mM $CaCl_2$; addition of 2mM $CaCl_2$ resulted in further reduction in the GT. pH cycling decreased the GT of heated milk with no added $CaCl_2$ and there was a further reduction in GT with addition of $CaCl_2$.

Rate of curd firming (K) and gel strength (G′)

Addition of $CaCl_2$ resulted in faster rates of curd firming and formation of stronger gels in both the heated and unheated milk systems. G′ values increased by 17% and 28%, respectively, upon addition of 1mM and 2mM $CaCl_2$ to the control milk. Added $CaCl_2$ had a marked effect on K and G′ of heated milks. G′ values increased from 2 Pa (no $CaCl_2$) through 58 Pa (1mM $CaCl_2$) to 86 Pa (2mM $CaCl_2$). Corresponding increases were also observed in K values. pH cycling of the heated milks before addition of

$CaCl_2$ at either of the 2 levels had a negligible effect on K and G′.

The accelerating effects of calcium chloride on the rennet coagulation of unheated milk are well documented (Mehaia and Cheryan, 1983a; van Hooydonk *et al.* 1986a; Singh *et al.* 1988) and $CaCl_2$ is frequently added to cheesemilk to enhance rennet coagulation during commercial cheese manufacture.

While there is still debate on the effect of Ca^{2+} on the primary (enzymic) phase of rennet coagulation of milk, it is well established that the secondary phase is completely dependent on a critical Ca^{2+} concentration (Pyne 1953). Therefore, the decrease in GT and increase in K and G′ observed in both the heated and unheated milk samples was probably due to increased aggregation of renneted micelles caused by the increased [Ca^{2+}]. van Hooydonk *et al.* (1986a) concluded that the degree of κ-casein conversion at RCT decreases with an increasing amount of $CaCl_2$ concentration and this too may account for the reduction in GT observed here. The exact mechanism of calcium action is, however, still unclear. It may be related to the charge reduction on casein micelles by binding of Ca^{2+} ions, thus reducing electrostatic resistance to aggregation of casein micelles (Green, 1982).

Addition of $CaCl_2$ causes not only an increase in calcium ion activity, but also increases the concentration of colloidal calcium phosphate (CCP). Holt *et al.* (1986) found that CCP as well as 'bound' Ca^{2+} were important with regard to the integrity of the casein micelles. Removal of CCP without affecting the serum distribution of calcium and inorganic phosphate has been shown to prolong the renneting process and this was attributed to structural changes in the micelles (Pyne and McGann, 1960).

Although pH cycling did improve rennet coagulation properties of heated milks, its effects at the extreme heating conditions used in this study were

very minor compared to those of $CaCl_2$ addition when the two were simultaneously carried out. This is clearly demonstrated in Figure 6.8 which is a plot of the change in storage modulus G′ with time after rennet addition. From this Figure, it can be seen that the rennet coagulation properties of the heated milk to which 2mM $CaCl_2$ was added were greatly improved and pH cycling of this sample before $CaCl_2$ addition had no significant effect on coagulation.

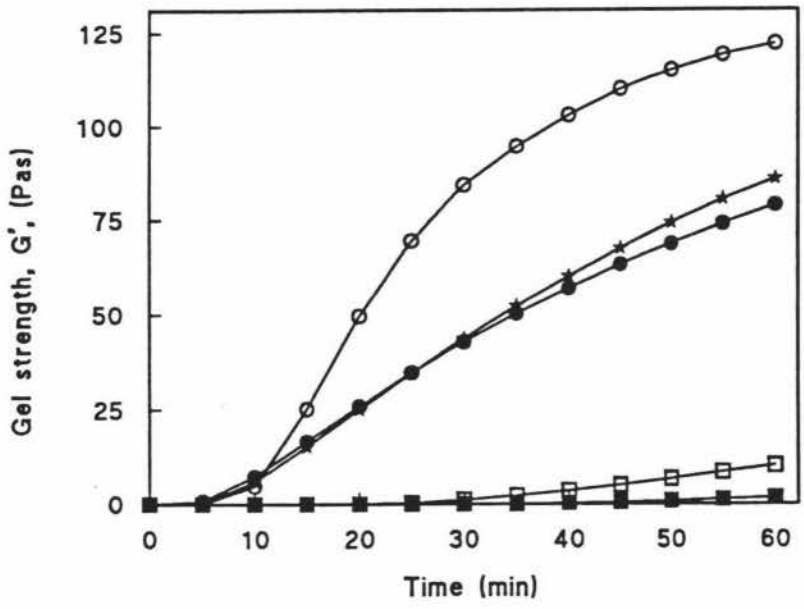

Figure 6.8: The combined effect of acidification and reneutralisation (pH cycling) and $CaCl_2$ addition on the changes of G′ with time during renneting of heated milk (140°C for 4 s). No $CaCl_2$ added- raw milk (control) (○), heated milk (■); heated milk with 2mM $CaCl_2$ added (▽); heated and pH cycled milk with no $CaCl_2$ added (□); or heated and pH cycled milk with 2mM $CaCl_2$ added (•).

6.3 Conclusions

The following general conclusions could be drawn from the results of the experiments described in this Chapter:

(i) pH cycling of heated milks (heated either at normal or alkaline pH) reduces the gelation time (GT) and increases the gel strength (G′).

(ii) Addition of increasing amounts of $CaCl_2$, at pH 6.5, significantly improves the rennet coagulation properties of heated and unheated milk.

(iii) pH cycling of heated milk before addition of $CaCl_2$ results in no significant further improvement in the rennet coagulation properties of heated milk.

CHAPTER 7

RENNET COAGULATION PROPERTIES OF MILKS CONCENTRATED BY ULTRAFILTRATION

The use of ultrafiltration (UF) is increasing in the dairy industry especially in the area of cheesemaking. UF has been successfully used for manufacture of soft varieties of cheese such as Feta, Camembert, Quarg, Cream and Cottage cheese (Kosikowski, 1986), but UF technology has not yet proved to be as beneficial in the manufacture of hard and semi-hard types of cheese such as Cheddar. One of the major problems encountered in the manufacture of hard and semi-hard cheeses from UF concentrated milks is that the products tend to be very hard and crumbly in texture. This hard texture problem of Cheddar type cheese manufacture may be associated with the difference in coagulation properties of UF milk because of high protein and calcium contents. This problem becomes more pronounced as the protein concentration increases. Kosikowski (1986) suggested that UF concentration of milk to a volume concentration ratio (VCR) of 1.8X is the limit for making good quality Cheddar cheese.

In Chapter 5, it was observed that heat-induced interactions between β-Lg and κ-casein increase the coagulation time and lower the gel strength of the curds after rennet addition. Heat treatment of UF milk may thus prove effective in correcting the curd firmness problem associated with hard cheese manufacture from unheated UF concentrates. Heat treatment of UF concentrates may also increase the yield of cheese by incorporation of whey proteins.

This chapter describes experiments carried out to determine:

(i) the influence of concentration of milk by UF on rennet coagulation properties.

(ii) the influence of UHT and pH cycling of UHT treated UF concentrates on rennet coagulation properties.

(iii) the influence of heat treatment of UF concentrates (3X) at different temperatures on rennet coagulation properties and the relationship between the state of whey proteins and the rennet coagulation properties.

7.1 Concentration of skim milk by UF

Skim milk was concentrated by UF to volume concentration ratios (VCRs) of 2X and 3X and analysed for total protein concentration and total solids as described previously (Chapter 4).

7.1.1 Changes in protein and total solids concentration

Table 7.1 shows the effect of UF on the pH, protein and total solids concentration (T.S.) of skim milk.

Table 7.1

pH, protein and total solids content of UF skim milk concentrates.

VCR	pH after UF	% Protein	% T.S.
Milk	6.72	3.10	9.07
2X	6.71	6.24	12.08
3X	6.68	8.90	14.61

UF increased the concentrations of protein and T.S. and the pH of milk decreased slightly with concentration. The final composition of the milk concentrates is dependent on the efficiency of the UF conditions used as well as on the integrity of the UF membrane. The concentrate composition obtained in these experiments were similar to those reported by previous workers (Mehia and Cheryan, 1983b; Lucisano *et al.* 1985).

7.1.2 Changes in rennet coagulation properties

Normal milk and milk concentrates obtained at VCR 2X and 3X were divided into 2 sub-samples one of which was adjusted to pH 6.5 and the other left at the natural pH before renneting. The coagulation process was followed as described previously (Chapter 4).

Figure 7.1A shows the rheological profiles of the gels formed from the UF concentrates during renneting at their natural pH values. For both the normal milk and UF concentrates, G′ had begun to rise at ~40 min after rennet addition. This was followed by a rapid increase in G′ with time. The increase in G′ with time was much more pronounced in the UF concentrates than in normal milk and in all cases G′ tended to approach a constant value after ~90 min. The shapes of the G′ versus time curves changed progressively with the initial slopes increasing with increase in VCR.

Adjustment of pH at renneting to 6.5 resulted in faster increase in G′ with time and a shorter time (~60 min) for the curves to reach a constant G′ value (Figure 7.1B). The rate of increase of G′ and the final value of G′ increased with VCR at both natural pH and at pH 6.5 (compare Figures 7.1A and 7.1B).

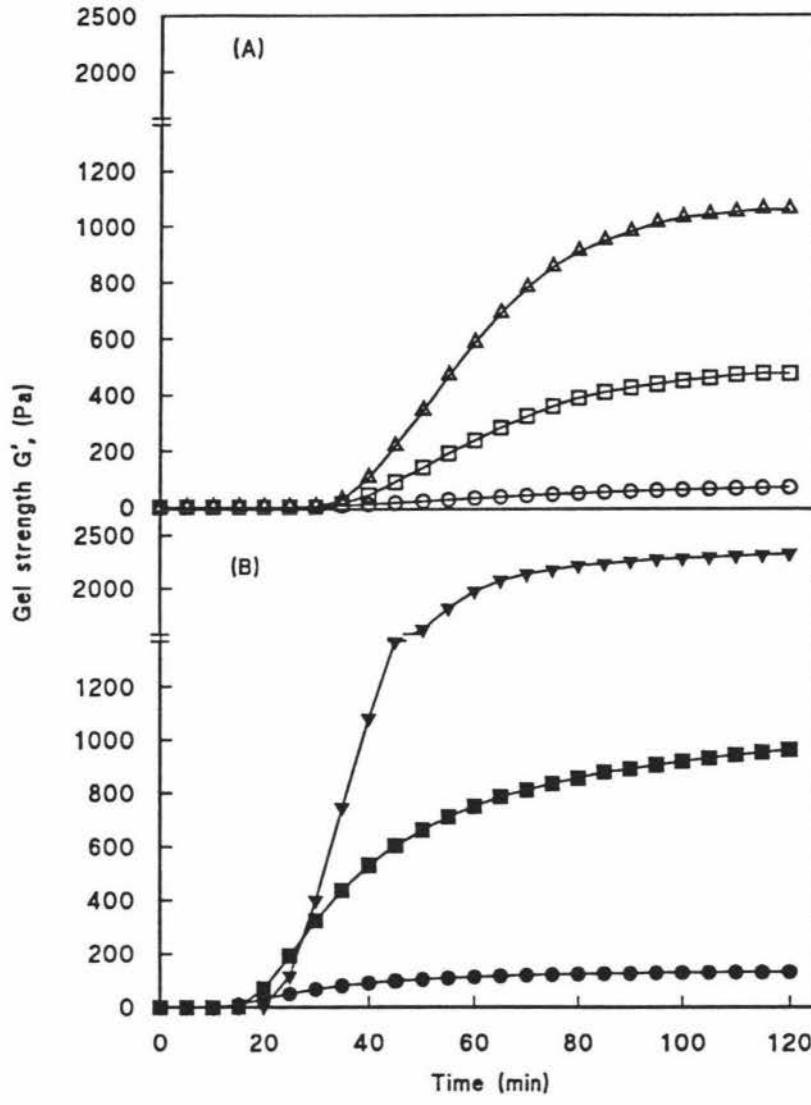

Figure 7.1: The changes in gel strength (G′) with time during renneting at (A) natural pH of normal skim milk (○), 2X UF concentrate (□) or 3X UF concentrate (△) and (B) at pH 6.50 of normal skim milk (•), 2X UF concentrate (■) or 3X UF concentrate (▼).

The effect of renneting pH on the GT, K and G′ (obtained from Figures 7.1A and 7.1B) of UF concentrates is shown in Table 7.2:

Table 7.2:

The effect of pH adjustment on the rennet coagulation properties of UF concentrated milks.

VCR	pH	GT	K	G′, (Pa)
Milk	6.72	24.5	1.08	69
2X conc.	6.71	21.5	9.30	474
3X conc.	6.68	22.5	22.96	1060
Milk	6.50	9.5	1.71	133
2X conc.	6.50	13.5	17.07	963
3X conc.	6.50	17.5	54.50	2320

Gelation time (GT)

Table 7.2 shows that at the natural pH of the milk concentrates, there was a slight decrease (from 24.5 to 21.5 min) in GT as the VCR was increased to 2X but no significant change in GT occurred with further increase in VCR up to 3X.

Adjustment of the concentrates to pH 6.5 caused GT to decrease markedly at all VCRs. At this pH, however, GT showed a linear increase with increasing VCR.

It is thus clear from the above that the trend observed in GT as a function of protein concentration (i.e. VCR) is at least partly dependent on the pH at which measurements are made. At the natural pH, there is little effect while adjustment to pH 6.5 results in GT increasing with increase in VCR.

The results obtained for GT at the natural pH are essentially in agreement

with those reported by Dalgleish (1980), who adjusted the pH of the concentrates to that of natural milk (pH 6.7) and observed no change in rennet coagulation time (RCT). Lucisano *et al.* (1985) determined the clotting time of UF concentrates at the same pH as used in this experiment (6.50) for the samples with adjusted pH. They obtained a sharp reduction in clotting time (measured using a modified Berridge method) as the protein concentration was increased from 3% to 11% and a constant amount of rennet added. Their results are in variance with those shown in Table 7.2 for the samples renneted at pH 6.5 where an increase in GT was observed. The discrepancy observed between the results (at pH 6.5) shown in Table 7.2 and those of Lucisano *et al.* (1985) is typical of the confusion that currently exists in the literature concerning the effect of UF on RCT. Some workers (e.g. Green and Morant, 1981; Mehaia and Cheryan, 1983b) have reported a reduction in coagulation time after UF while others (e.g. Garnot and Corre, 1980 and Chaplin and Green, 1980) have observed an increase. These differences have been attributed to the different methods used to evaluate RCT, different interpretations of the term RCT, the various experimental procedures and conditions, mechanical handling of milk and the precision and sensitivity of the methods used (Lucisano *et al.* 1985).

The gelation time in milk concentrates is influenced by three parameters: rate of enzymic reaction, amount of κ-casein to be hydrolysed for gelation to occur and rate of aggregation. These in turn are dependent on conditions such as pH, $[Ca^{2+}]$, and ionic strength as outlined in the literature review (Chapter 2). Most of these variables are substantially affected upon UF concentration, particularly the concentration and state of the proteins (especially casein), the equilibrium of minerals (especially calcium), and the pH. UF-induced changes in these alter rennet coagulation in terms of the kinetics of the reactions (for both the primary and secondary phases) and of the resulting curd structure (Garnot and Corre, 1980; Lucisano *et al.* 1985).

Adjustment of pH to 6.5, as carried out in this study, is more representative of the conditions used in the practical cheesemaking process, where rennet is added to UF concentrates after some degree of acidification through the metabolism of starter cultures.

Rate of curd firming (K) and gel strength (G′)

As expected, there was an increase in K and G′ of the rennet gels as the VCR increased at both the natural pH and at pH 6.5 of the milk and concentrates (Table 7.2). The values of K and G′ of the UF concentrates renneted at pH 6.5 were greater than those at natural pH (Table 7.2).

The results for K and G′ reported in this study are essentially in agreement with Culioli and Sherman (1978), Reuter *et al.* (1981), Green *et al.* (1981) and Green (1990) who demonstrated that the rates of firming of curds formed from UF milk increased in proportion to the extent of concentration of the milk used in cheese manufacture. The increase in K and G′ could be attributed to the higher casein micelle concentration in these concentrates which, once renneted, aggregate at a faster rate and form stronger links due to their closer proximity once the volume of the aqueous phase has been decreased by UF.

The increase in K with VCR observed is particularly important since it influences the determination of the appropriate time for curd cutting during cheese manufacture. The curd must be cut at the 'right' firmness to get good yield and a correct moisture level in the cheese. Therefore, although GT increased on renneting UF concentrates at pH 6.5, the time from renneting to curd cutting in manufacture of cheese from milk concentrated by UF would have to be significantly shortened due to the marked increase in K.

7.2 Ultra-high temperature (UHT) treatment of UF concentrates

Skim milk was ultrafiltered to VCRs of 2X and 3X, prior to heating at 140°C for 4 s. The heated samples were then divided into two sub-samples of which one was pH cycled (pH adjusted to 5.5, held at 20°C for 2 h and readjusted to pH 6.5) and the other adjusted to pH 6.5 before renneting as discussed in Chapter 4 (see flow chart below).

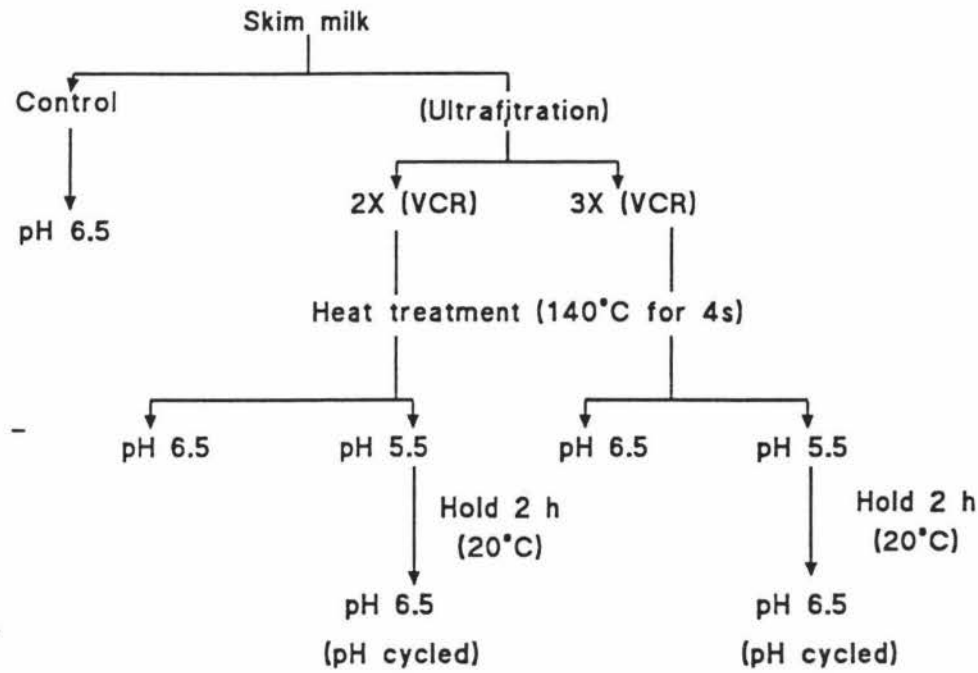

Figure 7.2: Experimental procedure for the effect of UHT treatment and pH cycling on the rennet coagulation properties of milk concentrated by UF.

Figure 7.3 shows the rheological profiles obtained upon renneting normal milk and the 2X and 3X UF concentrates (Figure 7.3a) and compares them with those obtained after UHT treatment of the milk and concentrates (Figure 7.3b).

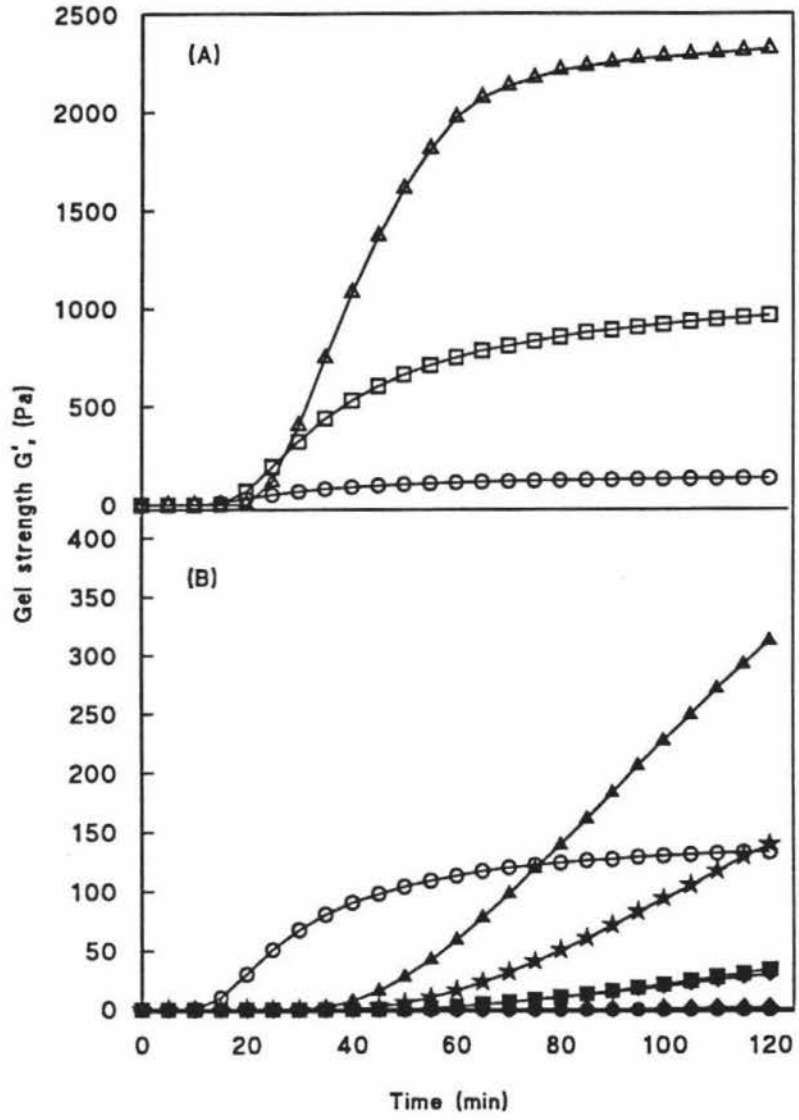

Figure 7.3: The changes in G′ with time during renneting at pH 6.5 of (A) unheated normal milk (○), 2X UF concentrate (□), 3X UF concentrate (△) or (B) UHT treated normal milk (•), 2X UF concentrate (■), 3X UF concentrate (▲) and UHT treated and pH cycled normal milk (◊), 2X UF concentrate (♦) or 3X UF concentrate (★). Unheated normal milk (○) is also shown in (B).

The G′ versus time curves for the unheated milk and UF concentrates were very similar to those described in Figure 7.1 (Section 7.1). The G′ began to increase within 20 min of renneting. For normal milk (control) the increase in G′ was fairly steady and the G′ versus time curve approached an almost constant G′ value after ~60 min. With increase in VCR there was a significant rise in the initial slopes of the curves.

As a consequence of heat treatment, the ability of normal milk to form a gel was severely retarded (Figure 7.3B). For the heat treated 2X UF concentrate, G′ increased only slowly with time. Heat treatment of the 3X UF concentrate markedly changed the shape of the G′ versus time curve compared to that of the unheated sample. The increase in G′ was quite fast and after ~70 min the G′ value was higher than that of unheated normal milk.

The effect of UF followed by UHT treatment (and pH cycling) of the UF concentrates on rennet coagulation properties is shown in Table 7.3. Table 7.3 also shows the effects of similar treatments on rennet coagulation properties of normal milk (control).

Table 7.3

The effect of UHT treatment of UF concentrates on their rennet coagulation properties.

VCR	GT (min)	K	G′, (Pa)
Milk NH	10.5	2.08	140
2X conc. NH	14.5	18.39	975
3X conc. NH	19.0	57.03	2354
Milk H	65.5	0.04	3
2X conc. H	37.0	0.53	34
3X conc. H	21.0	4.28	312
Milk H*	58.0	0.15	9
2X conc. H*	35.0	0.46	30
3X conc. H*	28.0	2.10	139

The sequence of process operations was UF → UHT → pH cycle. NB: NH = no heat, H = UHT treated, H* = UHT treated and pH cycled.

Gelation time (GT)

UF resulted in an increase in the GT of the unheated controls (Table 7.3). UHT treated normal milk had a GT >60 min. GT of the 2X concentrate increased (from ~15 to 37 min) after UHT treatment while that of the 3X concentrate was hardly affected by UHT treatment.

Rate of curd firming (K) and gel strength (G′)

UHT treatment of normal milk greatly impaired its ability to form a firm gel. K and G′ (after 2 h) also decreased in both the 2X and 3X concentrates as a result of UHT treatment (Table 7.3). The effects of UHT treatment on G′ became less severe as the VCR was increased and heated 3X concentrate still formed a gel that was stronger than that formed from unheated milk.

7.2.1 pH cycling of UHT treated UF concentrates

pH cycling before renneting had a negligible effect on the shapes of the G′ versus time curves of heated normal milk and 2X UF concentrate (Figure 7.3B). pH cycling of heated 3X UF concentrate, however, delayed the onset of gelation and greatly reduced the rate of increase in G′ over that of heated but not pH cycled 3X concentrate. G′ increased gradually with time until its value after 120 min was the same as that of unheated normal milk.

Gelation time (GT)

pH cycling of UHT treated normal milk decreased the GT to <58 min; there was a negligible change in that of the 2X UHT concentrate, while pH cycling of the 3X UHT concentrate caused an increase in GT from ~21 to 28 min (Table 7.3).

Rate of curd firming (K) and gel strength (G′)

pH cycling had small effects on K and G′ (after 2 h) in both heated normal milk and 2X UF concentrate. In contrast, both K (from 4.28 to 2.10) and G′ (from 312 to 139 Pa) were markedly reduced by pH cycling of the UHT treated 3X concentrate (Table 7.3).

7.3. UHT treatment of skim milk prior to concentration by UF

Skim milk was heated at 140°C for 4 s and divided into two sub-samples, A and B. Each sub-sample was then further sub-divided into three separate samples (C, D, E). Sub-samples D and E from A were ultrafiltered (2X and 3X) and then renneted (pH 6.5), while those from B were first pH cycled and then ultrafiltered to the same VCRs before renneting. Sub-sample C from A was renneted without any further treatments, while that from B was first pH cycled then renneted without any UF. This experimental procedure is shown diagrammatically below:

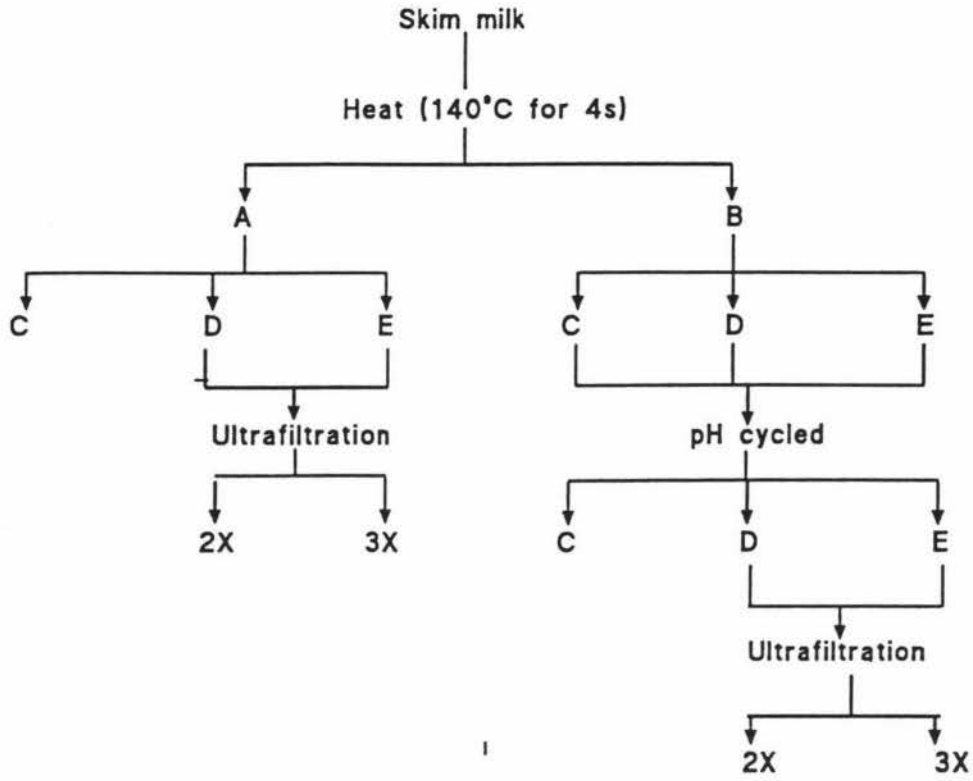

Figure 7.4: Experimental procedure for determination of the effect of concentration by UF on the rennet coagulation properties of UHT treated milk.

Figure 7.5 shows the effect of heat treatment of milk prior to concentration by UF on the rheological profiles obtained after renneting of the resultant UF concentrates. The shapes of the G′ versus time curves obtained from the heated milk and concentrates were different from that obtained from unheated normal milk. Heat treated normal milk showed negligible change in G′ with time during the 120 min over which measurements were taken. G′ for the heated 2X UF showed a slow increase in G′ with time. G′ for the heated 3X concentrate showed a fairly steep increase in G′ which intercepted that of normal milk after ~90 min. After 120 min, G′ of the heated 3X UF concentrate was higher than that of normal milk at the same time after renneting.

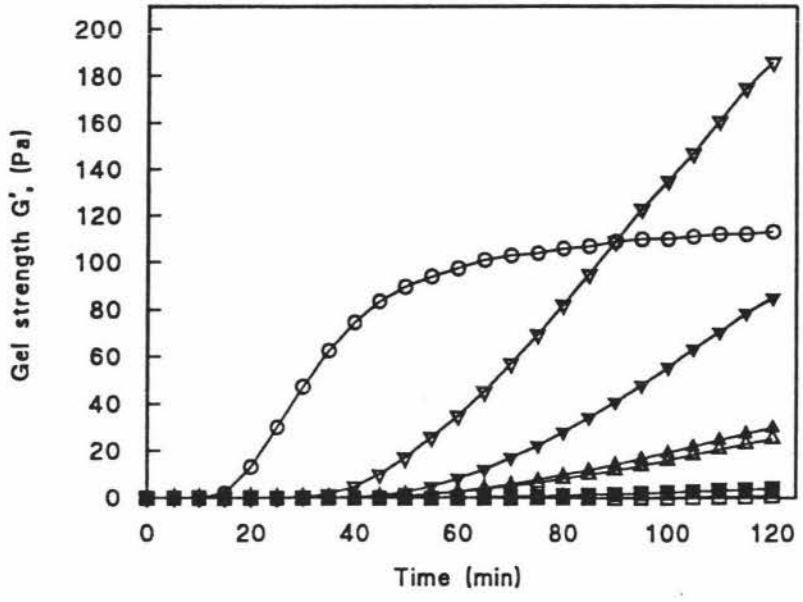

Figure 7.5: The changes in gel strength (G′) with time during renneting of unheated normal milk (○), normal milk UHT treated before UF to VCR 2X (△), or 3X (▽), normal milk UHT treated and pH cycled (■), normal milk UHT treated and pH cycled before UF to VCR 2X (▲) or 3X (▼).

The effect of the above pre-renneting treatments on GT, K and G′ are summarised in Table 7.4.

Table 7.4:

The effect of UHT treatment (and pH cycling) before UF on rennet coagulation properties.

VCR	GT (min)	K	G′ (Pa)
Milk NH	14.5	2.04	128
Milk H	73.0	0.02	1
2X conc. H	33.5	0.38	25
3X conc. H	25.5	2.43	186
Milk H*	57.5	0.70	4
2X conc. H*	34.0	0.37	25
3X conc. H*	30.5	1.31	85

The sequence of process operations was UHT → pH cycle (some samples) → UF. NB: in the table NH = no heat, H = UHT treated, H* = UHT treated and pH cycled.

Gelation time (GT)

In all cases, the GT of the UHT treated samples was longer than that of unheated normal milk (control). There was, however, a decrease in GT with increasing VCR for these samples (Table 7.4).

Rate of curd firming (K) and gel strength (G′)

Both K and G′ (after 2 h) values for the gels formed from UF concentrates prepared from UHT treated skim milk increased markedly as the VCR was raised (Table 7.4). The rennet gel formed from UHT treated skim milk concentrated by UF to VCR 3X was stronger than that formed from unheated normal milk.

7.3.1 pH cycling of UHT-treated skim milk prior to concentration by UF

pH cycling had no effect on the shape of the rheological profile obtained from heated normal milk (Figure 7.5). No significant change was detected on the G′ versus time curve obtained from the 2X UF concentrate prepared from heated milk that was pH cycled before UF when compared with that which was not pH cycled. pH cycling of heated milk before UF to 3X, however, had a marked influence on the shape of the resultant profile. The onset of increase in G′ was delayed and the initial slope of the curve was much lower compared to that of 3X UF concentrate prepared from heated milk that was not pH cycled. After 120 min, G′ for this sample was slightly lower than that of unheated normal milk.

Gelation time (GT)

pH cycling of UHT treated normal milk reduced its GT from 73 to 57 min but had a negligible effect on that of the UHT treated 2X concentrate; the GT of the heated 3X concentrate increased slightly as a result of pH cycling (Table 7.4).

Rate of curd firming (K) and gel strength (G′)

pH cycling immediately after heat treatment and prior to UF had little effect on both the K and G′ of normal milk and that of the 2X concentrate. There was, however, a marked decrease in K and G′ upon pH cycling of heated milk before UF to 3X VCR (Table 7.4).

7.4 Overall discussion on the effects of UHT and/or pH cycling before or after UF

Changes in casein micelle size distribution with increase in VCR have been suggested (Srilaorkul *et al.* 1991), but Lonergan (1983) observed no changes in size distribution, structure or composition of the micelles. The results of Lonergan (1983) were supported by those of Horne and Davidson (1993) who reported that it is unlikely that particle diameter changes with

the concentration of milk. Therefore, the most obvious effects of UF on the composition of skim milk are the increase in solids content due mainly to the selective concentration of the protein fraction and bound mineral salts (colloidal calcium and magnesium phosphates) and the decrease in the concentration of the soluble ions (Lonergan, 1983).

The increase in rate of curd firming and gel strength of both heated and unheated UF concentrates relative to that of normal milk is probably due to the high concentration of casein micelles in the UF concentrate. Concentration of the casein micelles decreases the mean free distance between them and increases the probability of the 'reactive sites' on adjacent renneted micelles fusing together to form bridges. The increased strength of rennet gels formed from UF concentrates may thus be a function of the increased number of bonds/bridges between renneted micelles and the stronger attractive forces (e.g. Van der Waals attractions, hydrophobic bonds and salt bridges) compared to the unconcentrated system. Because UF concentrated milks form very strong gels, the effects of UHT on the gel strength of milk are counteracted by UF treatment either before or after UF. It is not clear, however, why the gel strength of the rennet gels formed from milk that was heated before UF were considerably lower than those from milk that was concentrated before heat treatment. It is possible that the nature of the β-Lg/κ-casein complexes and the way in which these associate with casein micelles upon heating of UF concentrates is different from that in normal milk. In addition, the equilibrium of mineral salts in UF concentrated milks is different to that in normal milk and this may have an effect on how the two systems behave during heating and renneting.

The results presented above show that unlike in heated normal milk, pH cycling did not improve the rennet coagulation properties of heated UF concentrates regardless of whether it was undertaken before or after UF. In fact, pH cycling had a marked detrimental effect on rennet coagulation

properties of the 3X concentrate. This observation can be explained by consideration of the state of calcium in milk which is affected by changes in composition, such as pH, and technological treatments, e.g. heating and cooling. Ultrafiltration of milk induces a further element of change and uncertainty. During UF, colloidal calcium is fully retained in the concentrate, while the soluble calcium freely permeates through the membrane. Consequently, the colloidal calcium concentration increases, roughly maintaining a constant ratio with protein concentration (Casiraghi *et al.* 1987).

When heated milk was pH cycled before UF, there was presumably an increase in $[Ca^{2+}]$ in solution (Singh *et al.* 1988). During subsequent UF treatment of this milk, this $[Ca^{2+}]$ was lost in the milk permeate, resulting in poorer rennet coagulation properties (possibly due to reduced rate of aggregation in the secondary phase) of such treated milk when compared to a sample that was not pH cycled. This effect is similar to that observed by other workers (Covacevich and Kosikwoski, 1977; Lucisano and Casiraghi, 1985) who attempted to lower the gel strength and syneresis of rennet gels obtained from UF concentrated milk by acidification and\or diafiltration of milk prior to UF.

Since pH cycling of the concentrates after UF and heat treatment did not improve their rennet coagulation properties either (see Section 7.3.1), it appears that the explanations are more complex than proposed above but should involve the equilibrium of mineral salts (especially the calcium salts) and how these interact with and influence the nature of the associations between β-Lg and κ-casein.

It is apparent that a more thorough investigation is needed on the influence of technological treatments such as heating, UF and pH cycling before renneting on the equilibrium of milk salts as well as on the nature of associations between β-Lg and κ-casein.

7.5 Heat treatment of UF concentrates (3X) at different temperatures

Raw skim milk was divided into two samples of which one was concentrated by UF to a VCR of 3X. The milk and 3X concentrates were then separately heated at temperatures in the range 80 - 140°C for 4 s. Heated samples were then analysed for whey protein denaturation, association with casein micelles, incorporation into rennet gels, and rennet coagulation properties as described in Chapter 4.

7.5.1 Rennet coagulation properties of heat treated UF (3X) concentrates

The effects of heat treatment of 3X UF concentrates at temperatures in the range 80 - 140°C on the rheological profiles are shown in Figure 7.6. G′ began to increase within 20 min for all samples heated at ≤120°C. In these samples, the G′ versus time curves became progressively less steep as the heating temperature became more severe. This trend was similar to that observed for normal milk (see Chapter 5), except that G′ values for 3X concentrates were much higher.

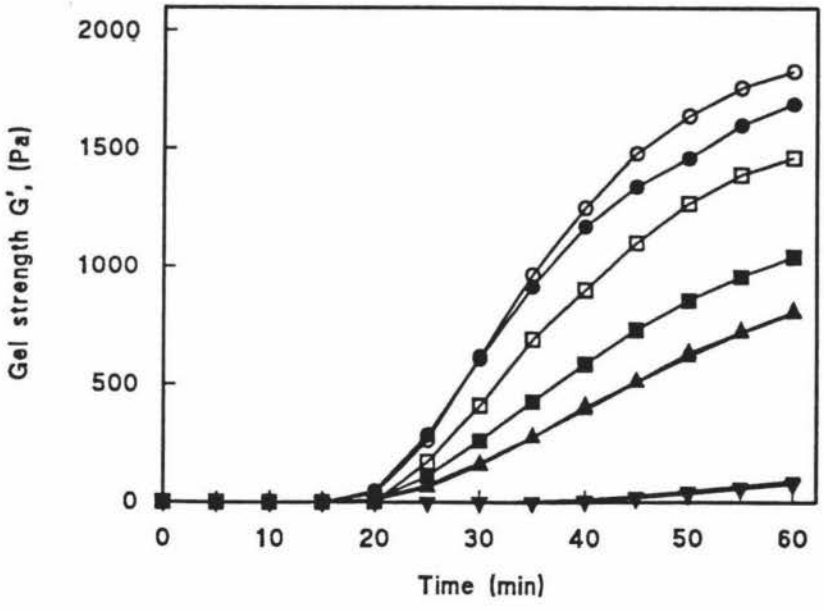

Figure 7.6: The changes in gel strength (G′) with time during rennet coagulation of heated 3X UF concentrates. Unheated control 3X UF concentrate (○), UF concentrate (3X) heated at 80 (•), 90 (□), 100 (■), 110 (△), 120 (▲), 130 (▾) or 140°C (▽) for 4 s.

The effects of heat treatment on GT, K and G′ of UF concentrates (3X) upon renneting are shown in Table 7.5.

Table 7.5

Effects of heat treatment of UF concentrates (3X) on (rennet) gelation time, rate of curd firming and gel strength

Heating Temperature (°C)	GT (min)	Rate of curd firming (K)	Gel strength (G′-Pa)
Control	16.0	47.49	1830
80	15.5	39.69	1640
90	16.0	37.86	1460
100	16.0	27.22	1040
110	16.0	21.97	805
120	16.0	15.40	498
130	24.0	3.60	91
140	28.0	3.55	77

Gelation time

The GT of the 3X concentrates remained constant at ~16 min up to a heating temperature of 120°C (Table 7.5) but it increased with further increase in temperature.

Rate of curd firming (K) and gel strength (G′)

Table 7.5 shows that there was almost a linear decrease in both K and G′ of the 3X UF concentrates with increase in heating temperature from 80 to 120°C. While normal milk heated at 130 or 140°C failed to form a gel of measurable strength (see Chapter 5), 3X UF concentrate heated at the same temperatures formed gels of strength comparable with those of unheated normal milk.

These observations are essentially in agreement with those of McMahon *et al.* (1993) who found a significant decrease in both the rate of gel firming and final gel firmness upon heating 3X UF concentrates at temperatures between 80 and 120°C. In the current study, 3X samples heated to temperatures of 130 or 140°C formed gels similar in strength to those formed from mildly heated normal milk and this may have potential in correcting the hard, crumbly texture problem encountered in hard cheese manufacture from UF concentrates.

7.5.2 Relationship between the state of whey proteins in the 3X UF concentrate and its rennet coagulation properties

Gelation time

Figure 7.7 relates the increase in GT with heat treatment to the amount of residual native or non-sedimentable β-Lg in the heated 3X UF concentrates. As the amount of residual native β-Lg in the 3X concentrate decreased down to ~20% of original, there was very little change in GT. However, with further decrease in residual native β-Lg below 20% GT increased very rapidly.

No change was observed in GT of the 3X concentrate with a decrease in non-sedimentable β-Lg down to ~40% of original. Further decrease in non-sedimentable β-Lg resulted in a sharp increase in GT.

In Chapter 5 it was concluded that up to 40% denaturation or 35% association of β-Lg had little effect on the onset of coagulation in normal milk. UF of milk to a VCR of 3X prolonged this limit to ~75% and ~60% denaturation and association of β-Lg, respectively.

Gel strength (G')

Figure 7.8 relates G' of heated 3X UF concentrates to the amounts of residual native or non-sedimentable β-Lg.

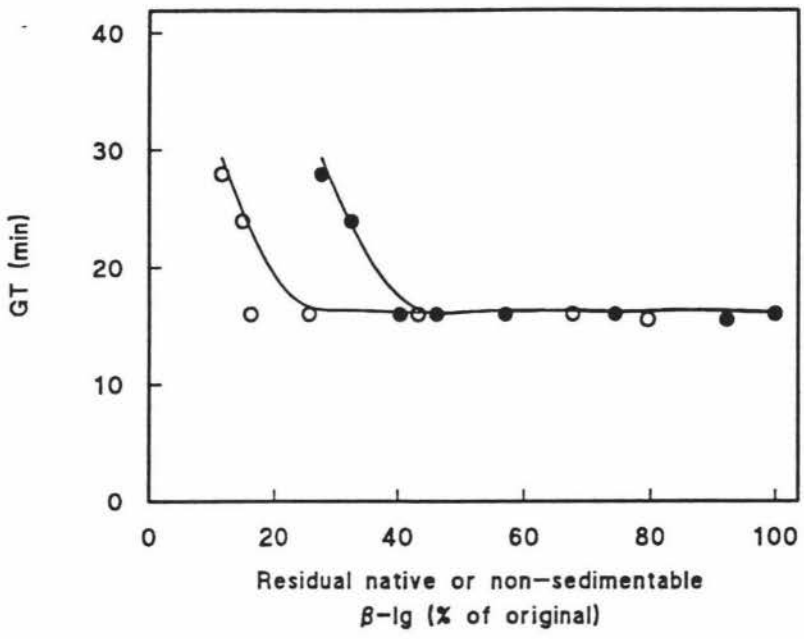

Figure 7.7: Relationship between residual native β-Lg (o), or non-sedimentable β-Lg (•) and gelation time in heat treated 3X UF concentrates.

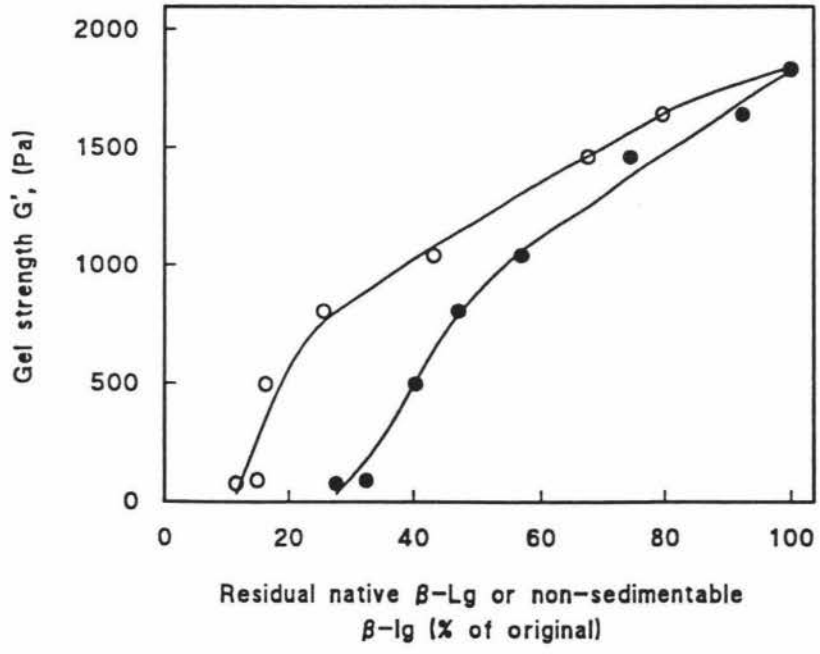

Figure 7.8: Relationship between the amounts residual native β-Lg (o) or non-sedimentable β-Lg (•) and gel strength (G') in heated 3X UF concentrates.

G′ decreased almost linearly as the amount of residual native β-Lg decreased down to ~25% (i.e. 75% denaturation) but further decrease in native β-Lg caused a sharp decrease in G′.

The relationship between G′ and non-sedimentable β-Lg was also not linear. As the concentration of non-sedimentable β-Lg decreased to ~45%, G′ decreased almost linearly, but the slope of the line increased markedly at lower levels of non-sedimentable β-Lg.

Comparison of the results presented in this Chapter with those in Chapter 5 reveals that concentration of milk by UF counteracts the effects of heat treatment on the rennet coagulation properties of milk since rennet gels could still be formed from severely heated UF concentrates. The gels formed from the UF concentrates were much firmer than their unconcentrated counterparts at comparable levels of β-Lg denaturation and association. This is probably because UF of milk increases the protein concentration and lowers the volume of the aqueous phase which lowers the mean free distance between casein micelles, and so increases the aggregation velocity of renneted micelles. This coupled with the fact that in UF concentrates there is a higher level of colloidal calcium results in coagulation being initiated at a lower extent of κ-casein hydrolysis (Garnot and Corre, 1980; Dalgleish, 1981; McMahon *et al.* 1993).

It was interesting to note in Figure 7.8 that there was a marked difference in gel strength between 3X concentrate samples with ~25% residual native β-Lg and those with less (~10 - 20 %). A similar observation was made by McMahon *et al.* (1993) who reported a large difference in gel strength between 3X milk samples heated at 123°C and 140°C despite a small difference in the extent of whey protein denaturation. They attributed this to a difference in the nature of the material coating the micelle surfaces after heating at 120°C and at 140°C. This difference, they suggested, could be a result of irreversible denaturation of α-La or further changes in the

structure of β-Lg which exhibits a second thermal change near 140°C with breakdown of disulphide bonds and additional unfolding (Watanabe and Kostermeyer, 1976). It could also be a function of the precipitation of insoluble calcium phosphate that occurs during heating (Mohommad and Fox, 1987). McMahon *et al.* (1993) further proposed that the accumulation of extraneous protein on the casein micelles during UHT processing of 3X skim milk is a two-step process. Initially, a compact mass of material (presumably denatured β-Lg) complexes with κ-casein on the surface of the casein micelles in milk heated at temperatures between 89 and 123°C. Additional protein material is then adsorbed on the layer of β-Lg upon heating to 140°C, to form a loose coating encircling the micelles. This secondary layer of protein was thought to have the most detrimental effect on enzymic milk coagulation. This may explain the observation made in the current study of a large difference in gel strength between 3X UF concentrates with ~25% residual native β-Lg and those with less.

7.6 Conclusions

The results presented in this Chapter led to the following conclusions:

(i) The trends observed in the influence of UF on the onset of gelation (GT) are dependent on the pH at which renneting is carried out.

(ii) The rate of curd firming and strength of rennet gels increases with increase in VCR of milk concentrated by UF.

(iii) Firm gels may still be formed from milk heated at temperatures up to 140°C for 4 s before or after UF although the gelation time increases and gel strength decreases with the severity of the heat treatment.

(iv) pH cycling of heat-treated UF concentrates reduces the gel strength; milks concentrated to higher VCRs being more adversely affected.

(v) Heat treatment of milk before UF results in weaker gels being formed on subsequent renneting compared to heat treatment after UF.

(iv) Firm rennet gels may still be prepared from UF concentrates containing up to 75% denatured β-Lg.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 Mechanisms of rennet gel formation

The casein micelles in normal milk show no tendency to aggregate due to their stability which has been attributed to two possible mechanisms. First, micelles have a negative surface charge that is partly due to the C-terminal part of κ-casein (Donnelly *et al.* 1984; Dalgleish *et al.* 1989). Stability then arises because of repulsion of these like charges and is explained by DLVO theory (Green, 1973). A second mechanism (favoured by a number of authors e.g. Walstra, 1979; Darling and van Hooydonk, 1989) is that of steric stabilisation, whereby the hydrophillic moieties of κ-casein protrude into the aqueous solution (whey) from the surfaces of the micelles as flexible 'hairs'. Aggregation of the casein micelles is prevented because the 'hairy' outer layers of the micelles cannot interpenetrate.

By hydrolysing the κ-casein on micellar surfaces into para-κ-casein and a glycomacropeptide (GMP) during the enzymic phase of renneting, rennet provides reactive sites ('hot spots') depleted of κ-casein *via* which the micelles can aggregate. The rate of aggregation increases with increase in temperature and calcium concentrations. Van der Waals and hydrophobic interactions as well as ionic linkages (involving Ca^{2+}) are thought to be involved in the aggregation process. Electron microscopic observations show that micelles aggregate to form chains, which then link together to form a network. Initially, micelles are linked by bridges which then contract bringing the micelles together and eventually causing partial fusion (Green *et al.* 1978). These processes progressively strengthen the links between micelles. Thus, both the number and strengths of the links increase with time. These characteristics are possibly responsible for the rise in G′ or curd firmness after the gelation time (Figure 5.9). This

mechanism of rennet curd formation in raw milk is depicted diagrammatically in Figure 8.1.

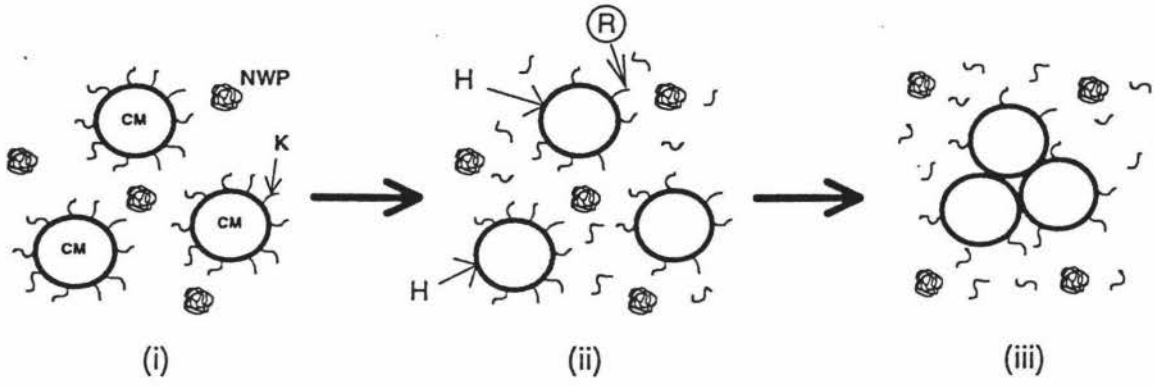

Figure 8.1: Schematic diagram of the attack on casein micelles (CM) by rennet (R). (i) CM are stabilised against coagulation by hairy layer of κ-casein (K). Native whey proteins (NWP) are also shown. (ii) R attacks K on CM surfaces creating 'hot spots' (H). (iii) Renneted CM aggregate through the 'hot spots' to initiate gel formation. NWP play no part in the reaction at any stage.

Heat treatment of milk at temperatures >70°C alters the basic mechanism of rennet coagulation outlined above resulting in increased coagulation times and weaker gels (Morrisey, 1969; van Hooydonk *et al.* 1987; Singh *et al.* 1988; Lucey *et al.* 1993a). The formation of a complex between β-Lg and κ-casein *via* sulphydryl-disulphide interchange on heating milk (Zittle *et al.* 1962; Sawyer *et al.*1963; Sawyer, 1969; Dalgleish, 1990b) is believed to be the most important factor affecting rennet coagulability of milk (Kannan and Jenness, 1961; van Hooydonk *et al.* 1987; Dalgleish 1990a). This complex increases the stability of casein micelles since the denatured whey proteins attached to the micellar surface further shield the micelles against aggregation. Although the mechanism of the influence of the β-Lg/κ-casein complex on the renneting reaction has not been fully elucidated, a number of factors may be involved.

(i) formation of a complex on the micelle surface may increase surface

charge and hydration (Singh and Fox, 1985; Schmidt and Poll, 1986). This would cause an increase in electrostatic repulsion between the enzyme molecules and casein micelles. Consequently, the enzyme may not be able to hydrolyse sufficient κ-casein on the micelle surfaces to make them aggregable, since it is known (Dalgleish, 1979; Chaplin and Green, 1980) that a considerable proportion of the κ-casein must be hydrolysed before aggregation at a measurable rate can occur.

(ii) Complex formation may also reduce aggregation of casein micelles even if it has no influence on κ-casein hydrolysis (Marshall, 1986). By attaching to the surfaces of the micelles, denatured whey proteins may initiate steric repulsion which may hinder the close approach of the renneted micelles. After κ-casein conversion, aggregation will take place mostly between micelles not fully covered with whey proteins. The interaction forces between the sites rich in denatured whey proteins are likely to be too weak to form a strong gel. Thus the number and strengths of the contacts between adjacent aggregate chains within the gel network, are likely to be decreased as a result of whey protein complexation, resulting in poorly developed gels. The effect increases as heating temperatures become more severe.

(iii) Other factors such as decrease in ionic calcium and formation of heat-induced colloidal calcium phosphate (van Hooydonk *et al.* 1987) may also affect the rate of aggregation and gel formation of renneted micelles.

The effects of heat treatment on the rennet coagulation properties of milk are summarised in Figure 8.2 which may be compared to Figure 8.1.

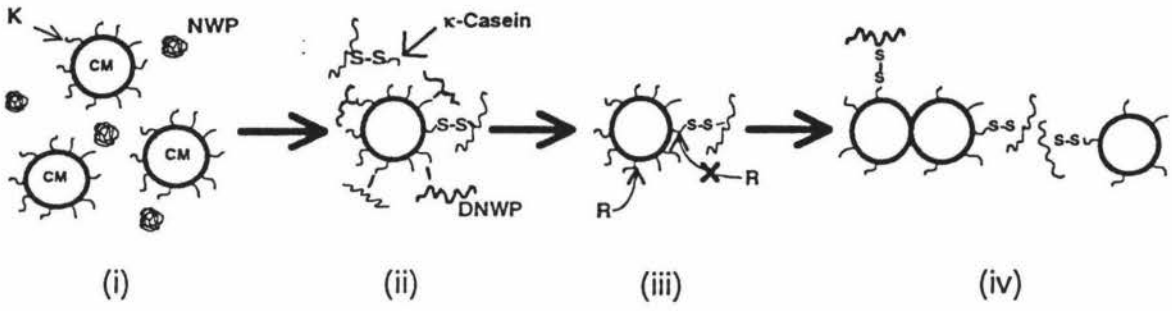

Figure 8.2: Schematic diagram of the effect of heat treatment of milk on the attack of casein micelles (CM) by rennet (R). (i) CM are stabilised against aggregation by a 'hairy layer' of κ-casein (K). (ii) heat treatment converts the native whey proteins (NWP) to denatured whey proteins (DNWP) which associate with both micellar and serum κ-casein. (iii) κ-casein hydrolysis is either prevented or retarded due to presence of denatured whey proteins on the micellar surfaces. (iv) denatured whey proteins on micellar surfaces reduces the number of 'hot spots' and also sterically hinder renneted micelle aggregation.

8.2 Restoration of coagulation properties of heated milks

Provided that the heat treatments applied to the milk have not been too severe, the adverse effects of heat treatment on rennet coagulation of milk may be at least partially reversed by decreasing the pH, adding $CaCl_2$ or pH cycling. Lowering the pH results in increased $[Ca^{2+}]$, solubilisation of heat-induced calcium phosphate and increased rennet activity. Addition of $CaCl_2$ reduces the pH of milk and increases the $[Ca^{2+}]$.

Adjustment of the pH of heated milk to values <6 followed by readjustment to the natural pH (pH cycling) may partly offset the adverse effects of heating (providing that heat treatment has not been too severe) on rennet coagulation of milk (Banks, 1988; van Hooydonk *et al.* 1987; Singh *et al.* 1988; Imafidon and Farkye, 1993; Lucey *et al.* 1993a). The effect of changing the pH in this way is to partly dissociate the calcium phosphate

of the micelles as the pH is lowered (Davies and White, 1960; Dalgleish and Law, 1989) followed by its reformation (and possible partial reconstruction of the micelles) as the pH is raised again, although not all the dissociated [Ca^{2+}] is restored to the micelles as the pH is increased (Singh *et al.* 1988).

The common effect of these three processes is an increase in [Ca^{2+}]. The aggregation of para-casein micelles is dependent on a critical amount of Ca^{2+} (Pyne and McGann, 1960) and above this the RCT of milk decreases with increase in the amount Ca^{2+} (Walstra and Jenness, 1984). By increasing the [Ca^{2+}], these treatments increase the rate of aggregation of renneted micelles and so help initiate bridging and chain formation between the micelles. The spatial distribution of casein aggregates and the strengths of intra- and intermicellar bonds may also be affected by $CaCl_2$ addition. This could cause an increase in gel strength as observed in Section 6.2. Direct addition of $CaCl_2$ is obviously more effective in increasing the [Ca^{2+}] than pH cycling and this explains the greater efficiency of $CaCl_2$ addition in restoring the rennet coagulation properties of heated milks (see Chapter 6). pH cycling may only provide sufficient [Ca^{2+}] to initiate bridging and network formation between renneted micelles, but perhaps not enough to influence subsequent stages of gelation during which the network extends and becomes more differentiated as the bonds strengthen. It is suggested, therefore, that even after pH cycling, casein micelle surfaces would still be covered with denatured whey proteins thus limiting the micellar surface area that is completely bare of κ-casein (due to proteolysis during the primary phase) and denatured whey proteins. As a result, fewer and weaker linkages between micelles would occur leading to weak gels despite reduced GT (see Figures 6.1 and 6.2) in heat-treated pH-cycled milk.

8.2.1 Influence of concentration by UF

Concentration of milk by UF increases the number of casein micelles per given volume of milk. This causes a slight reduction in the rate of κ-casein hydrolysis, probably because of the decrease in the effective diffusion rate of the enzyme molecules (van Hooydonk *et al.* 1984). The number of 'reactive sites' created by rennet also increases with concentration and there is a decrease in the mean free path between micelles. This may result in increase in the number of 'effective collisions' (i.e. those between reactive sites leading to bridging) between para-casein micelles (Sharma *et al.* 1990; McMahon *et al.* 1993). The number and strength of bonds within the gel network and the rate at which they are formed also increases resulting in higher rates of curd firming and stronger gels. The basic mechanism of rennet gel formation in heated UF concentrates is presumably similar to that proposed for heated milks (Figure 8.2) except the effects of heat treatment are compensated by the increase in casein micelle concentration.

8.3 Implications in cheese manufacture and product development

By heat treatment of milk at temperatures causing denaturation of the whey proteins, there is potential for increasing the yield of cheese per unit of milk. However, maximum benefits in yield may be obtained at temperatures milder than those used in UHT operations since good correlation between associated β-Lg and that incorporated into the rennet gel was obtained at extents of association <50%. More severe heat treatments (>120° for 4 s) cause irreversible changes in rennet coagulation without any additional whey proteins being incorporated (see Figure 5.8b). Good rennet coagulation properties may be most effectively restored in heated milks by addition of $CaCl_2$ at pH ~6.5. The proportion of rennet retained in cheese curd is strongly related to the pH of the curd at draining (Holmes *et al.* 1977; Creamer *et al.* 1985) and is believed to influence cheese flavour development. At pH 6.5 there should be minimal enzyme retention and the cheese should be of acceptable organoleptic

quality.

Heat treatment of milk either before or after concentration by UF lowers the rates of curd firming and gel strengths of unheated UF concentrates. Similarly, pH cycling of 3X concentrates either before or after UF also weakens the strengths of the resultant gels. This has potential in the production of UF concentrates with controlled gelling properties suitable for semi-hard and hard cheese production with improved texture (i.e. smoother, finer, less crumbly cheeses). Heat treated UF concentrates could also be spray dried to produce milk protein concentrate (MPC) powders with desired rennet coagulation properties for export to countries with low milk production or where milk supply is very seasonal. In the importing country the user would need only to add water, starter and rennet to make a cheese-type product. However, further research is needed to determine the microstructure of the renneted curds formed from UHT treated 3X UF concentrates and how this would handle and develop during subsequent cheesemaking steps such as cutting and ripening.

The work discussed in this study on the effect of heat treatment and pH cycling of heated UF concentrates as well as the sequence of these operations should initiate new ideas in the use of UF concentrated milks and MPC powders for the manufacture of different varieties of cheese. It must, however, be emphasised that this research should be approached more with the aim of new product development as opposed to trying to emulate the flavours and textures of existing products as is the current trend.

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