EFFECT OF TRANSGLUTAMINASE ON MILK PROTEINS

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XIMENITA ISABELLE TREJO ARAYA

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ABSTRACT

In this study transglutaminase was used to achieve ε-γ-(glutamyl) lysine cross-linking of milk proteins, in Trim™ and Full Fat milks, the same milks with a variety of added protein concentrates, and finally in yogurt and Petite Suisse acid gel systems. The effects of a preheat treatment, enzyme incubation temperature, enzyme inactivation after the enzyme incubation period and homogenization on the cross-linking of the three major casein and two whey proteins were also studied. The degree of cross-linking was established by the use of SDS PAGE gel electrophoresis.

The results indicated that cross-linking of the major casein and whey proteins was maximized if the milk was preheated for 10 minutes at 90°C and then cooled before addition of the transglutaminase. However, the preheat treatment was not always advantageous in Trim™ milk systems, but was essential for Full Fat milk systems. Maximal cross-linking of milk proteins occurred if the enzyme/milk system was incubated at 37°C for two hours rather than at 55°C for the same period. The extent of cross-linking increased in an almost linear fashion with increasing transglutainase concentration in most milk systems, with maximal cross-linking occurring when the enzyme concentration was 100 U/mL. Studies on one milk system showed that whey loss and gel strength deteriorated if more than 100 U/mL of enzyme was used.

The study demonstrated that homogenization was an essential step for protein cross-linking if the system contained any fat. Casein and whey protein transglutaminase mediated cross-linking was maximized in Full Fat milk systems if the milk was homogenized before transglutaminase was added. Maximal cross-linking, particularly of whey proteins, occurred in Full Fat milk systems if the milk was preheated for 10 minutes at 90°C, cooled to 60°C and then homogenized at 50/150, cooled further to 37°C and then incubated with 100 U/mL of enzyme for two hours.

Addition of sodium caseinate or milk protein isolate to Trim™ and Full Fat milk systems was shown to significantly improve protein cross-linkage by up to 50% for β-casein and whey protein respectively. Transglutaminase addition to milk systems containing the previously mentioned protein concentrates further enhanced cross-linking compared to the non-enzyme controls, particularly when the enzyme concentration was 100 U/mL

Addition of transglutaminase to acid milk gels dramatically improved the whey holding and gel properties of the products, particularly when the enzyme concentration was 100 U/mL. The reduction in whey loss was proportional to transglutaminase concentration up to 100 U/mL. A 100% reduction in whey syneresis and a 10g F improvement in gel strength improvement were obtained when 0.5 % sodium caseinate and 100 U/mL of transglutaminase were added to a gel milk system compared to a control sample with no enzyme. The physical properties of the milk acid gels were further improved if the transglutaminase in the acid gel systems was not inactivated prior to the addition of the enzyme.

The addition of milk protein concentrates such as sodium caseinate and total milk proteinate were shown to have dramatic effects on the whey holding and gel properties of acid gels. Moreover, the properties showed little reduction over a two week storage period compared to yogurt with no added protein. The addition of transglutaminase at a concentration of 100 U/mL further enhanced the above physical characteristics of the acid milk gels. Variations in cross-linking within systems containing either sodium caseinate, milk protein concentrate and milk protein isolate were observed. These variations need to be examined in further work. The addition of NaCNTMP further enhanced the gel and whey-holding properties compared to systems containing either sodium caseinate or total milk proteinate.

The final study was conducted on Petite Suisse, a high fat acid milk gel, and here the addition of transglutaminase at 100 U/mL dramatically improved the gel strength of the system by 500% compared to the control.

Finally, this research confirmed that transglutaminase effectively cross-linked milk proteins, and in particular β- and κ-casein and β-lactoglobulin.

Transglutaminase addition to milk and acid milk systems clearly improved some of the physical properties of the systems. However, much work is needed before it could be recommended for use by industry. The effect of adding transglutaminase to acid milk gels and milk systems should be evaluated by consumer panels to ensure that the sensory properties of these systems have in no way been compromised. Furthermore the economic costs of adding transglutaminase should be determined to ensure that the process would not

be uneconomic. If the above evaluations prove to be beneficial then the process could be investigated and further studies carried out to see whether improvements could result by addition of transglutaminase to such milk products as yogurts, desserts, cheese etc, and to create new products with different textural and water holding characteristics.

Further work is needed on a scientific front to assess the effects of transglutainase and added proteins on the structure of milk gels and the precise mechanism of filament formation in these gels. Some questions were also raised concerning the exact mechanism that was responsible for removal of monomeric forms of whey protein in the various milk systems evaluated in this study, and these should be determined by further research work.

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CHAPTER I

1.0 INTRODUCTION

Proteins are important components of food for human nutrition and are widely used as functional ingredients for improving the texture or viscoelastic properties of foods (Sakamoto et al., 1994).

The functional properties, e.g. viscosity, water-holding capacity, gelation, mouthfeel, and emulsifying and foaming properties, are closely related to a protein's molecular structure and interaction; it is therefore of great importance to increase our knowledge of the relationship between protein structure and functionality (Faergemand, 1998).

Protein gelation plays a major role in the preparation and acceptability of dairy products (Boye et al., 1995). Gels made from milk protein are traditionally formed by thermal denaturation of the whey proteins or by treating caseins with acid or a proteolytic enzyme (chymosin). In milk protein gels, the resulting network structure is typically held together by non-molecular physical cross-links, electrostatic interactions, hydrogen bonding and hydrophobic bonds (Dickinson and Yamamoto, 1996).

An alternative way of making a milk protein gel could be by enzymatically cross-linking the protein molecules to produce a network of covalent linkages. This new protein network might have different rheological properties from a conventional milk protein gel (Dickinson and Yamamoto, 1996).

The enzyme transglutaminase can catalyze an acyl transfer reaction between γ-carboxymide groups of glutamine residues and ε-amino groups of the lysine residues of peptide chains, giving as a result a covalent ε-(γ-glutamyl) lysine bond between protein molecules. Thus transglutaminase could have great potential for improving the physical properties of many foods without affecting the sensory properties of the product such as flavor and odor.

The aim of our study was to investigate the interaction between transglutaminase, at different concentrations and thermal conditions, and milk proteins and protein concentrates.

Once the optimal conditions were defined, transglutaminase was applied to yogurt manufacture with the objective of improving the texture and reducing syneresis.

CHAPTER II

2.0 LITERATURE REVIEW

2.1 Milk Proteins

Milk production is much more efficient than meat production in terms of the mass of protein that can be produced per hectare, but is much less efficient in this respect than plants such as soybeans and cereals. However, the nutritional and functional properties of milk are superior. For this reason, milk proteins have been a popular topic for research over the past 30 years (Fox, 2001).

Milk proteins can be divided into caseins and serum or whey proteins. Nearly 80% of milk protein is comprised as caseins. They are defined as phosphoproteins, which precipitate at pH 4.6, whereas serum proteins are soluble under such conditions.

The caseins exist as colloidal aggregates, containing thousands of protein molecules, whereas serum proteins are not aggregated in this way but are free in solution (Dalgleish, 1982).

Caseins are more complex proteins than serum proteins because they have important metal ion binding capabilities, and as a result their precipitation reactions are more complex. Serum proteins are typically globular proteins, and have important properties when denatured (Dalgleish, 1982).

The caseins can be divided into five fractions: the α_{s1}- (38%), α_{s2}- (10%), β- (36%), κ- (13%) and γ- (3%) caseins. The γ-caseins is a separate group, produced from β-casein by the proteolysis of milk by proteases (mainly blood fibrinolysin) (Dalgleish, 1982).

The serum protein group contains mainly β-lactoglobulin (54% of the total serum protein) and α-lactalbumin (21%) and the remainder, which is composed of serum albumin, immunoglobulins and protease-peptones (Dalgleish, 1982).

2.1.1 Caseins

The caseins are the predominant milk proteins of almost all mammalian species. They are phosphoproteins, and are present as stable calcium phosphate protein complexes called micelles (Rasmussen et al., 1999).

The bovine caseins are important food proteins and are widely used as additives in numerous food products (e.g. in baked products, cereals, coffee creams, desserts and pasta products). Caseins and their derivatives have also been employed in a variety of

pharmaceutical applications (Christensen et al., 1996). In addition, caseins have a biological function in that they provide the progeny with a source of phosphate and calcium for the mineralisation of calcified tissues, as well as amino acids and biologically active peptides (Rasmussen et al., 1999).

Four types of bovine caseins, i.e. the α_{s1}-, α_{s2}-, β- and κ-caseins, are characterised by size and structure. They are relatively small molecules, with a molecular mass of 20–25 kDa. Experimental techniques indicate that the caseins have low levels of secondary and tertiary structure, although theoretical calculations indicate that they do have some degree of higher structure. It has been suggested that, rather than lacking secondary structure, the caseins have very flexible, unstable structures and have been described as 'rheomorphic' (Fox, 2001). The lack of stable secondary and tertiary structure renders the caseins stable to denaturing agents, i.e. heat or urea, and contributes to their high surface activity, which gives them good foaming and emulsifying properties and renders them readily susceptible to proteolysis (which is important in cheese ripening and in the production of protein hydrolysates for dietary applications).

The caseins are relatively hydrophobic but have high surface hydrophobicity (because of their open structures) rather than a high total hydrophobicity. The hydrophobic, polar and charged residues are not uniformly distributed throughout the molecular sequences but occur as hydrophobic or hydrophilic patches, giving the caseins strongly amphipathic structures that make them highly surface active (Fox, 2001).

2.1.1.1 Casein Micelle

Caseins are found with calcium phosphate in large colloidal micelles of about 100 nm in radius. The casein micelles maintain the calcium phosphate in a soluble and bioavailable state and thereby enable the transport of calcium. Calcium phosphate is essential in the mineralisation of bones and teeth. For neonates, the main source of calcium is milk and human adults in western populations obtain up to 75% of their required calcium from milk products (Rasmussen et al., 1999).

The casein micelle is sterically stabilised by a layer (or 'brush' or 'hairs') of κ-casein molecules. This 'brush', which is a polyelectrolyte, is part of the κ-casein molecule which extends into the surrounding liquid and carries groups charged by salt ions in solution (de Kruif, 1999). The stability of the 'brush' depends on factors such as pH, brush density, salt concentration etc. (de Kruif, 1999).

The micelles are very stable, and can be frozen and even dried. Their properties will not be changed if reconstituted. Therefore, if we compare this colloidal system with other (synthetic) colloidal systems, we can state that casein micelles are extremely stable (de Kruif, 1999).

The micelles are strongly hydrated and have a large voluminosity of about 4.4 mL/g protein. The micelles comprise 93% (w/w) casein with the α_{s1}- to α_{s2}- to β- to κ-caseins in the weight ratio 3:1:3:1 (Schmidt, 1980; cited by Wong et al., 1996).

In the absence of calcium, polymers of different sizes will be linked to the hydrophilic bonds between non-polar groups. In the presence of calcium, perfect micelles are formed (Schmidt, 1980; cited by Wong et al., 1996).

Thus, it is possible to change the state of casein micelles by activating calcium ions and breaking the equilibrium of the stable form (Alais and Linden, 1991).

Caseins can be precipitated by concentrations of calcium > 6 mM. As bovine milk contains ≈ 30 mM calcium, it might be expected that the caseins in milk would precipitate. However, κ-casein, which contains only 1 mol of PO_4 per mol of protein, is insensitive to calcium ions and, moreover, can stabilise up to 10 times its weight of the calcium-sensitive caseins against precipitation by calcium. It does this via the formation of a type of quaternary structure, referred to as the casein micelle (Alais and Linden, 1991).

The micelles are composed of sub-micelles (Figure 1). The core of the sub-micelles is considered to consist of the calcium-sensitive α_{s1}-, α_{s2}- and β-caseins, with variable amounts of κ-casein located principally on the surface of the sub-micelles. The κ-casein-deficient sub-micelles are located in the centre of the micelles whereas the κ-casein-rich sub-micelles are concentrated at the surface (Morr, 1973; cited by Hillier and Lyster, 1978). Micelles can remove free sulphydryl groups from β-lactoglobulin through complex formation with κ-casein. The smaller micelles have more κ-casein, and have been shown to be less stable, than the larger micelles, even though they contain higher concentrations of the heat-induced β-lactoglobulin-κ-casein complex. This suggests that casein micelles have a stabilising effect (Hillier et al., 1979).

To summarise the characteristics of casein, Walstra (1999) proposed a model, which has been suggested for decades and is based on the structure of the bovine casein micelle (Figure 1). Some of the elements of the model are: "the casein micelle is roughly spherical, but not with a smooth surface; it is built in smaller units (sub-

micelles), which contain caseins; the sub-micelles can be linked together by small calcium phosphate clusters bridging them; the casein sub-micelles aggregate until they have formed a micelle, where those with κ-casein are at the outside. As a consequence, molecular chains of the C-terminal and of κ-casein protrude from the micelle surface, forming a 'hairy' layer that prevents by steric and electrostatic repulsion, any further aggregation of sub-micelles." The hairy layer is responsible for the stability of the micelles against flocculation.

The casein micelles can be affected by the addition of protein concentrates. Studies carried out in yoghurt stabilised with milk proteins (Modler and Miloslav, 1982) indicated that, when skim milk powder or milk protein concentrate was added as a stabiliser, the casein micelles were held together in chains by short thin links. When sodium caseinate was added as a stabiliser, the casein micelles appeared to be larger and more fused, compared with the use of the other milk proteins as stabilisers.

In yoghurts stabilised using whey protein concentrate (WPC), the casein micelles appeared in the form of individual entities surrounded by finely flocculated protein. Fine protein floccules, about one-tenth the diameter of casein micelles, occurred only in yoghurts fortified with WPC, and were probably composed of whey proteins denatured during the heating of the initial milk mix prior to inoculation with bacterial lactic cultures. Modler and Miloslav (1982) also indicated that the β-lactoglobulin added to the milk mix in the form of WPC might be able to react with the κ-casein of the casein micelles during heat treatment and form an insoluble complex. This could explain the accumulation of floccules on the surface of the casein micelle and then the formation of linkages between the micelles.

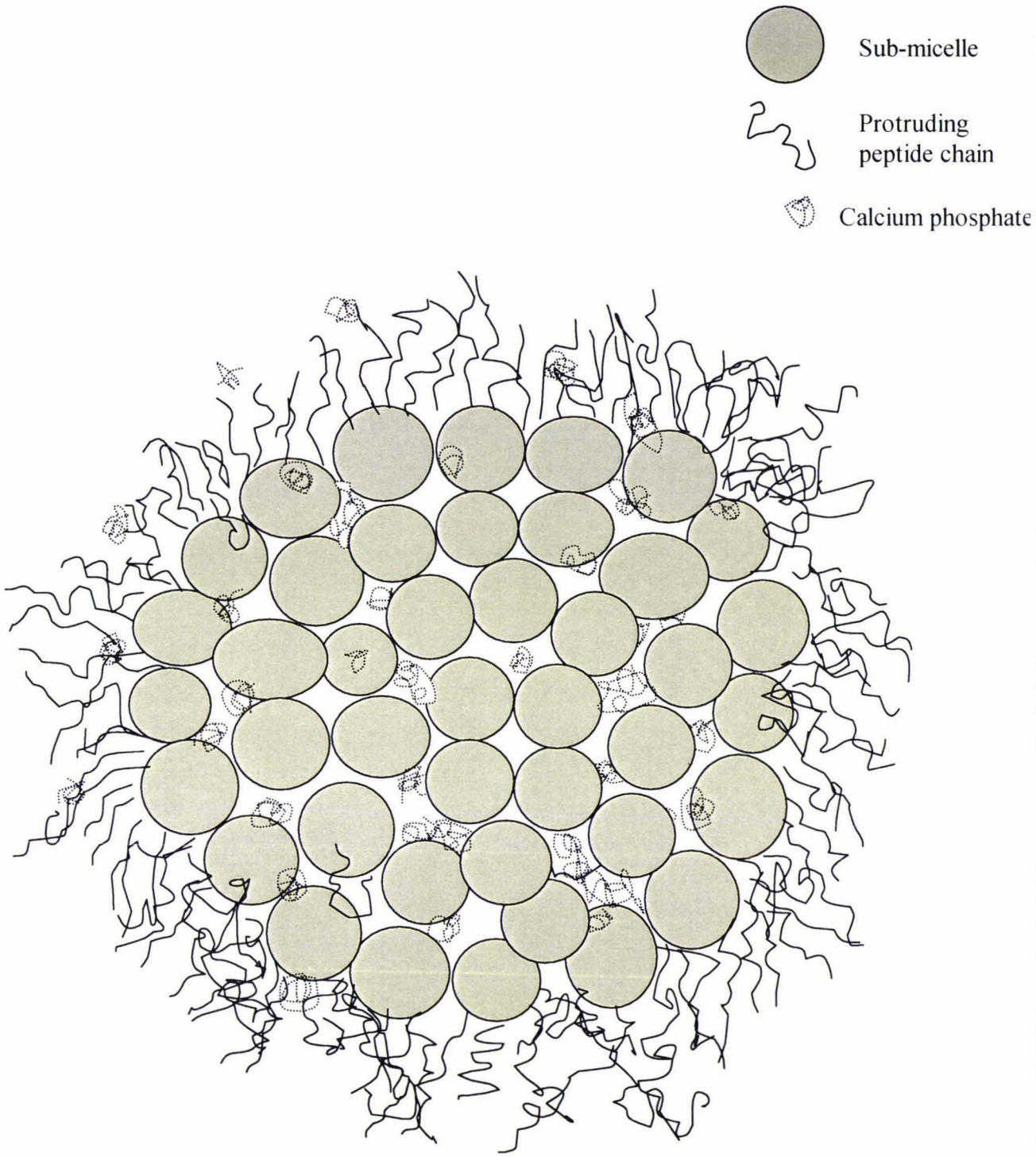

Figure 1: Model (cross section) of the casein micelle (from Walstra, 1999).

2.1.2 Whey Proteins

Whey is the soluble fraction of milk that is separated from the casein curd during cheese manufacture (Aguilera, 1995). The main whey proteins in bovine milk are: β-lactoglobulin (54%), α-lactalbumin (21%), blood serum albumin (BSA, 10%) and immunoglobulins (Ig, 10%; mainly IgG_1, with lesser amounts of IgG_2, IgA and IgM).

Protein products derived from whey proteins, such as concentrates and isolates (approximately 70-80 and 90% protein, respectively), have important commercial applications in gels (Aguilera, 1995).

The principal whey proteins are well characterised. In contrast to the caseins, the whey proteins possess high levels of secondary, tertiary and, in most cases, quaternary structure. They are typical globular proteins and are denatured on heating (90°C for 10 min). They are not phosphorylated and are insensitive to calcium ions. All whey proteins contain intramolecular disulphide bonds that stabilise their structure. β-Lactoglobulin contains one sulphydryl group that is buried within the molecule in the native protein but which becomes exposed and active on the denaturation of the protein by various agents (including heat). It can then undergo sulphydryl–disulphide interactions with itself or another protein (Fox, 2001).

β-Lactoglobulin is the major whey protein component of ruminant animals and that of many other mammals as well. It is present as a dimer in aqueous solution at pH values between 5.5 and 7.5 (Galani and Owusu Apenten, 1999). At temperatures above 40°C, the dimers split to form monomers, and this monomeric form is the predominant species in heat-denatured milk.

In contrast, α-lactalbumin is the smallest and the most heat-resistant whey protein (de Wit and Klarenbeek, 1984).

2.2 Protein Gelation

In the food industry, especially the dairy industry, protein gelation plays a major role in the preparation and acceptability of products.

A food gel is an interaction between a continuous phase of interconnected particles and/or macromolecules and another continuous liquid phase such as water. Depending on the type and concentration of the gelling agent, the salt content and pH of the aqueous phase and the temperature, gels can possess various degrees of rigidity, elasticity and brittleness. Some gels can be melted (liquefied) and reset with the

addition or removal of thermal energy, and these are designated as thermo-reversible gels. However, gels with covalent bonds between the molecules or complex particles are generally thermo-irreversible (Fennema, 1976).

To describe the mechanisms of gelation, Fennema (1976) explained that a sol can be transformed into a gel under various conditions, such as temperature change, chemical alteration of the gelling agent, reduction in the number of charged groups by adjustment of the pH or addition of salt and addition of water or the addition of a competitive compound, such as a sugar. In the transformation of sol to gel, a three-dimensional network is formed by the interaction of specific groups on polymer chains or particles to form cross-linkages at the sites of junction zones. The structure formed holds the aqueous phase. Different bonds are found in the junction zones: electrostatic, hydrophobic, covalent and hydrogen. "Thermo reversible gels have a preponderance of intermolecular hydrogen bonds, whereas in protein gels a few disulfide linkages per polymer chain may be sufficient to render them thermo irreversible." (Fennema, 1976).

Denaturation is the first step of gelation this process changes the three-dimensional structure of the native protein without breaking the peptide bonds.

When protein–protein interaction occurs, we are talking about aggregation. This aggregation can form high molecular weight complexes.

If the complexes are aggregated in a random form with a polymer–solvent interaction, we are talking about coagulation (Fennema, 1976).

In general, the differences between gelation, aggregation and coagulation are that gelation involves an ordered three-dimensional matrix whereas aggregation and coagulation involve more random complexes (Hudson, 1992).

Before describing the characteristics of milk gels, it is important to understand the terms native and denatured proteins, in the context of functionality.

In biochemistry, native means the state in which the three-dimensional conformation of a protein is in its natural environment. The denatured state (unfolding) is when a protein changes its conformation, which in turn affects its biological functionality (Franks, 1988; cited by Dickinson and McClements, 1995). "A fully unfolded protein has an approximately random coil structure which rapidly fluctuates between a very large number of different conformations, in contrast to the folded native protein, which has a definite single average conformation." The unfolded protein is thus more flexible, less compact and more hydrated than the folded protein. In practice, proteins

rarely unfold to a completely random coil conformation, and so some secondary structure is present even in the denatured state (Dickinson and McClements, 1995).

Gelation is a two-step mechanism: the first step involves denaturation of the protein, where it changes from its native conformation and unfolds. The second step involves the reorientation of the denatured proteins into a three-dimensional network (Hudson, 1992). This step is critical because it involves gel formation and will determine whether a gel, a coagulum or an aggregate is formed. If the change from the denaturation step to the reorientation step is fast, an opaque gel generally forms. However, if this occurs more slowly, the gel will be a fine network, less opaque and more elastic, and may exhibit less syneresis (Hudson, 1992).

In milk, gelation can be achieved by acid coagulation, enzyme action, heating and even storage. Most of these processes involve mainly the caseins, but interactions with β-lactoglobulin and α-lactalbumin also exist (Hunziker and Trassuk, 1965; cited by Wong et al., 1996).

Traditional milk protein gels include cheese, yogurt and dairy desserts. In these products, gelation is mainly due to the gelation/coagulation of casein (Mulvihill and Fox, 1989; cited by Faergemand, 1998). In cheese, for example, coagulation is induced by destabilization of the casein micelles because of the highly specific hydrolysis of κ-casein, thereby lowering the zeta potential and reducing the steric stabilization of the micelle, resulting in the coagulation of casein (Faergemand, 1998). In yogurt, gelation is a result of the acidification of milk due to the formation of lactic acid from lactose in the milk by the bacterial culture (Heertje et al., 1985; cited by Faergemand, 1998). Lowering of the pH to 5.5 releases calcium phosphate giving as a result of a partial disintegration of the casein micelles, which at normal milk pH will link the casein into micelles together again. Subsequently a re-aggregation of the casein will occurred when lowering of the pH to 5.2 and this will probably be due to charge neutralization of β-casein at its isoelectric point (Faergemand, 1998). At this stage, colloidal calcium phosphate in the casein micelles solubilizses and aggregates (Damodaran and Paraf, 1997).

2.2.1 Gelation of Caseins

Caseins have a high content of phosphoseryl residues, which permit them to bind polyvalent ions strongly, especially calcium ions, which promotes aggregation. Casein gels can be formed by treatment with rennet or by acidification or heating (Damodaran and Paraf, 1997).

During acidification, the 'hairy brush' (i.e. the fragment of κ-casein extending out from the micelle) becomes neutralized, collapses and releases calcium phosphate. The casein molecules (mostly β- and α-casein) leave the micelle as their isoelectric point is reached and exceeded. They then become positively charged and are re-integrated into the still negatively charged micelle. The modified micelles no longer repel other micelles and start to aggregate, with the consequent formation of networks and ultimately gel formation (Schorsch et al., 2000).

Caseins can gel by acidification, as mentioned above, or spontaneously during the storage of UHT-sterilized milks (age gelation). There is not much information about this phenomenon but it could be due to microbiological and/or physico-chemical reactions.

Proteases secreted by psychrotrophic bacteria could be the major causative factor in the spontaneous gelation of raw milk of low microbiological quality.

Another possibility of gelation is a dissociation of whey protein from casein micelles in UHT milk during storage, promoting dissociation of the micellar κ-casein and therefore gelation (Damodaran and Paraf, 1997).

2.2.2 Gelation of Whey Proteins

Whey proteins are functional ingredients in food and the effect of heat treatment on their functional properties is essential to a variety of applications of these proteins.

Heat-induced gelation is the major texturising procedure for most foods containing globular proteins such as egg white, soybean and whey proteins (Galani and Owusu Apenten, 1999).

Most globular proteins tend to aggregate when heated, but the aggregation mechanism can vary. The structure of the aggregates can also vary, not only between different proteins but also under different conditions (Le Bon et al., 1999).

When whey proteins are in a strong electrostatic repulsion form, the gels formed are transparent and have a fine stranded structure, whereas whey protein gels become more opaque and coarse with bigger pores under conditions of weak electrostatic repulsion (Langton and Hermansson, 1992; cited by Puyol et al., 2001). The gelation of whey proteins is shown in Figure 2.

Globular proteins have a charge that depends on the pH. Therefore, the electrostatic interactions will influence not only the kinetics but also the structure of the aggregates, and this explains the important influence of pH and ionic strength (Le Bon et al., 1999) on the gel properties, but we must not forget that temperature and protein concentration may also be parameters that influence the aggregation mechanism, which is described below.

2.2.3 Factors Affecting Gelation

As mentioned above, the texture and the strength of protein gels are affected by intrinsic factors such as the composition and concentration of the proteins and by extrinsic factors such as the heating temperature, pH, ionic strength and presence of other food components, e.g. lipids, sugars and starches (Boye et al., 1995) (Figure 3).

WPC gels can be altered by changing the factors that control the secondary structures of the individual whey proteins during gel formation (Boye et al., 1995). The heat-mediated gelation of a protein in water can be described as a two-stage process: the denaturation of the native protein, followed by protein–protein interactions resulting in a three-dimensional protein network that forms the final gel structure.

The most important factors in gelation are the heating temperature and the protein concentration (Taylor et al., 1994). If one of these factors is too low, then gelation will not occur.

Heat treatment can activate buried sulphydryl groups by unfolding proteins to expose these groups. These groups can then form new intermolecular disulphide bonds (bridging), which are required for the formation of a highly ordered gel structure in some protein systems.

Alteration of the temperature and time can affect the macroscopic and microscopic structural attributes of a gel, by changing the rates and mechanisms of denaturation and aggregation.

Research on WPC by Boye et al. (1995) indicated that, at temperatures above 70°C, very little of the protein was detected in sodium dodecyl sulphate polyacrylamide gel

electrophoresis (SDS-PAGE) gels, suggesting extensive aggregation at these higher temperatures. The β-lactoglobulin bands were not affected until 70°C. At temperatures below 65°C, no gelation was observed. However, an increase in the viscosity of the WPC dispersion was noted at this temperature, the temperature at which α-lactalbumin denatures. Gelation was observed only above 70°C, very close to the β-lactoglobulin denaturation temperature of 73°C.

The gelation of WPC, when heated above 70°C at neutral pH, could be attributed to molecular changes in β-lactoglobulin and only minimally to changes in α-lactalbumin. BSA and α-lactalbumin, which are at low concentration, could have aggregated first but were not incorporated into the gel matrix until β-lactoglobulin denatured (Boye et al., 1995).

Other important factors are the pH and the salt concentration, which can affect the balance of polar and non-polar residues. Protein–protein interactions are generally favoured under conditions that reduce the net charge on the molecules, e.g. pH values near the isoelectric point. High ionic strength tends to reduce electrostatic repulsion between proteins because of the shielding of ionisable groups by mobile ions (Ziegler and Foegeding, 1990).

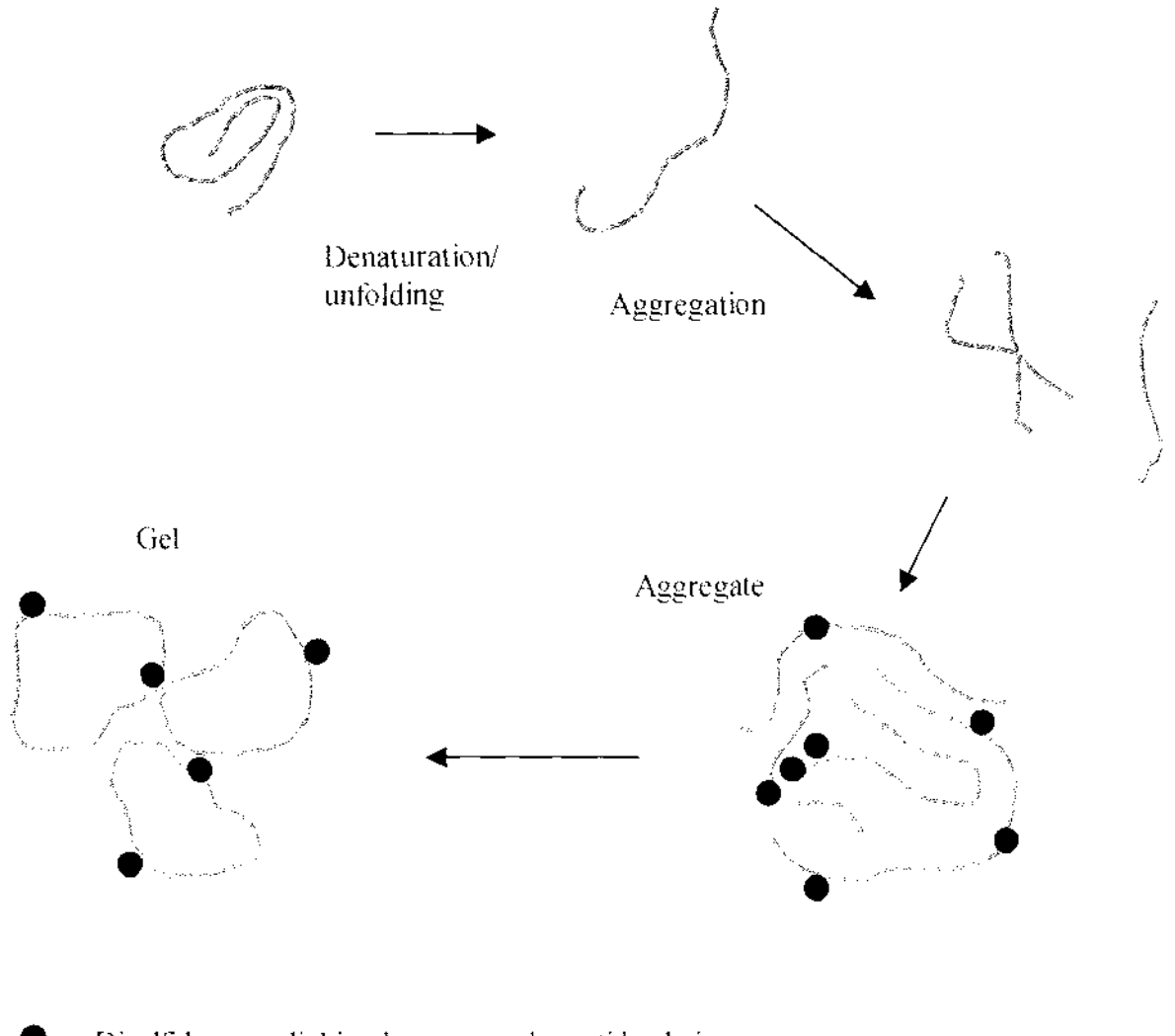

Figure 2: Schematic of the gelation of whey proteins (Aguilera, 1995)

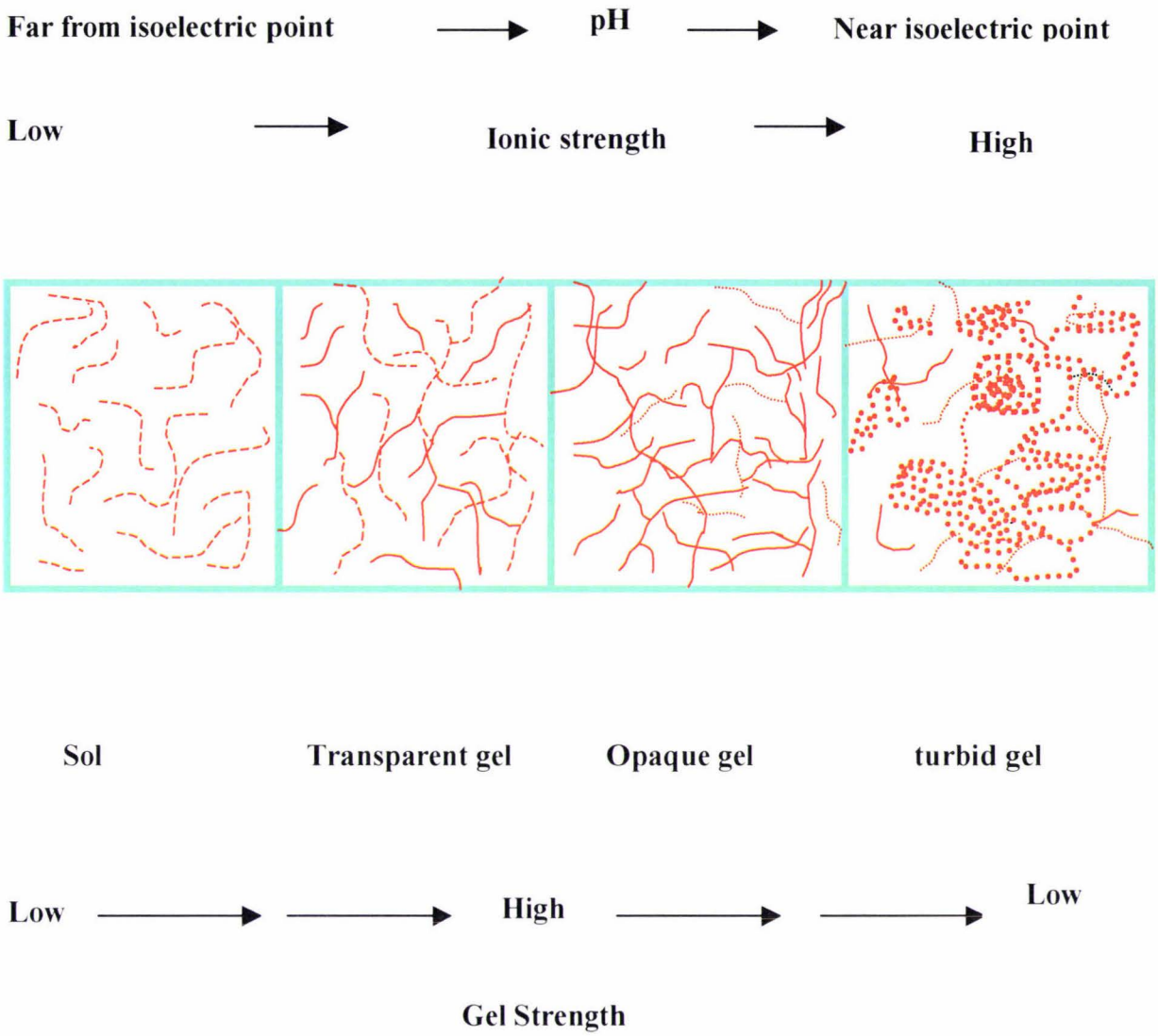

Figure 3: Model of the formation of transparent and opaque gels (from Damodaran and Paraf, 1997).

Changes in pH and/or salt concentration will affect the characteristics of whey protein gelation by interfering with the solubility, heat stability and protein–protein interactions during gel formation.

If the pH is close to the isoelectric point, proteins become less charged and less stable and, as a result, denaturation and/or aggregation occur more strongly and this can reduce the gelation time (Faergemand and Murphy, 1997).

When the pH is near the isoelectric point and the ionic strength is low, denatured proteins randomly aggregate by hydrophobic interaction. At a pH far from the isoelectric point and at low ionic strength, electrostatic repulsive forces hinder the formation of random aggregates, resulting in the formation of linear polymers (Doi et al., 1989; cited by Boye et al., 1995).

Firm and clear gels can be obtained between pH 7 and pH 10. In this region, the whey proteins can be expected to undergo extensive denaturation and expansion and to entrap more water. At acid pH (2–3), the gels are less firm (Boye et al., 1995).

In general, lowering the pH of WPC appeared to inhibit unfolding of the protein and increased its stability to thermal denaturation. A high pH enhanced the unfolding and subsequent aggregation of WPC. The unfolding of β-lactoglobulin resulted in the formation of intermolecular hydrogen-bonded β-sheet structures at both low and high pH.

Boye et al. (1995), when studying the effects of protein concentration and temperature on the gelation of whey proteins, showed that no gelation occurred below 65°C; no matter what concentration of whey protein was used. This could be related to the fact that protein denaturation is a prerequisite for gelation.

At low whey protein concentration (80 mg WPC/g H_2O), gelation occurred only at temperatures ≥ 75°C and the gel was very soft compared with solutions with higher protein concentrations. At concentrations lower than 80 mg WPC/g H_2O, no protein gelation occurred and only protein aggregates were formed. This suggested that the formation of gels from protein aggregates is dependent on the protein concentration.

Boye et al. (1995), suggested that, for a given type of protein, a critical concentration is required for the formation of a gel and also that the type of gel formed at a given temperature varies with the protein concentration.

Whey protein products, unlike casein, are stable to pH precipitation but are labile to heat. Heating causes unfolding and disulphide exchange between whey proteins. The

product obtained from the heat precipitation of whey is commonly marketed as 'lactalbumin', which is in fact a mixture of whey protein aggregates. The lactalbumin is totally insoluble and possesses a sandy and gritty mouth feel at high concentrations. The product is most useful for protein fortification in pasta, cereal, biscuits and bakery items (Wong et al., 1996).

2.3 Transglutaminase and its Food Applications

From a consumer perspective, texture is one of the most important attributes of food and has a major impact on the acceptability of a food.

From a psycho-physical perspective, texture is an extremely difficult and complex area. Investigations in this area involve the study of the rheological properties of a food, and the rheological relationship to the sensory perceptions of the food is largely dependent on the bonds that are formed within and between protein aggregates. These bonds can be one or more of the following: hydrogen, electrostatic etc.

In complicated systems such as meat, the texture is related to different cross-linking mechanisms, including ε-(γ-glutamyl) lysine cross-links formed through adol condensation reactions. These bonds are responsible for the formation of strong but elastic tissues in living organisms (Singh, 1991). This adol condensation could also be used in the fabrication of texturised products (Sakamoto et al., 1995).

It is important to remember that we are constantly required to increase the quality of food products, and it has become very useful to modify the properties of food to achieve this objective. An interesting possibility is to use enzymatic modification to improve the functional properties and nutritional value of a food (Motoki and Nio, 1983). The advantage of enzymatic modification over the traditional chemical techniques is that the reaction conditions can be milder and proteins can be modified at selected sites.

For example, transglutaminase is an enzyme that can hydrolyse proteins, add or delete various functional groups or promote intermolecular cross-links. It can also change both the rheological and surface-active properties of a food (Kinsella and Whitehead, 1989; cited by Dickinson and McClements, 1995). For example, it can convert a liquid protein into a viscoelastic emulsion gel, increasing the thermal stability and water-holding capacity. It can also improve the surface activity of a protein by attaching non-polar groups to the side chains of certain amino acids (Dickinson and McClements, 1995).

Transglutaminase is widely distributed in nature and has been found in various animal tissues, fish and plants (Kuraishi et al., 2001).

One transglutaminase that has been isolated from *Streptoverticillium* sp. is now used in the food industry. This microbial transglutaminase is active over a wide range of temperatures and is stable between pH 5 and pH 9, which is the pH range for most food processing. It is not reliant on calcium induction, as is mammalian transglutaminase (guinea pig liver transglutaminase) (Ajinomoto, 1997).

Transglutaminase catalyses an acyl group transfer between γ-carboximide groups of peptide-bound glutamine residues (acyl donor) and primary amino groups in a variety of amine compounds (acyl acceptor), including peptide-bound ε-amino groups of lysine residues. As a result of this cross-linking of peptide-bound glutamine and lysine residues via ε-(γ-glutamyl) lysine formed peptide bonds (Figure 4); high molecular weight protein polymer aggregates are obtained. In addition, transglutaminase is capable of catalysing the deamidation of glutamine residues, whereby water is used as a nucleophile and ammonia is liberated (Ikura et al., 1992; cited by Lorenzen and Schlimme, 1997).

Transglutaminase is much less selective toward the amine donor's lysine residues in proteins than it is toward the glutamine substrate (Christensen et al., 1996).

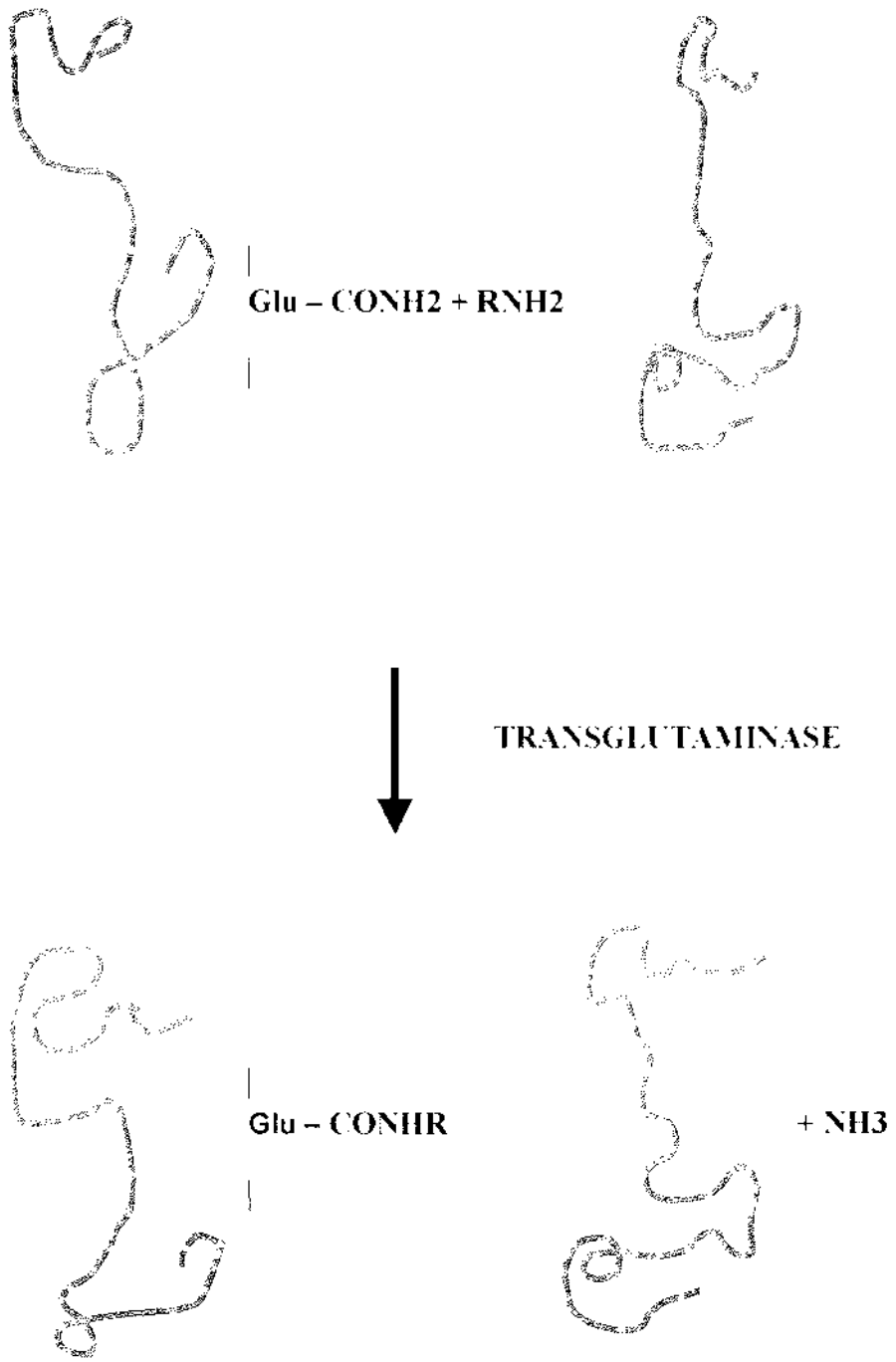

Figure 4: Cross-linking reaction between protein-bound glutamine and lysine by transglutaminase (from Nielsen, 1995).

"Generally, protein substrates of transglutaminase are classified into four groups: (1) Gln-Lys-type, in which both Gln and Lys residues are available for cross-linking; (2) Gln-type, in which only the Gln residue is available for reaction; (3) Lys-type, in which only Lys residues are available; (4) a nonreactive type, in which both Gln and Lys residues are unavailable for reaction (Ikura et al., 1984; cited by Han and Damodaran, 1996). This classification is mainly based on the accessibility of Lys and Gln residues located on the protein's surface." "According to the above classification, a mixture of two Gln-Lys-type substrate proteins or a mixture of Gln-type and Lys-type substrate proteins should be able to form heteroconjugates in a transglutaminase-catalyzed reaction. However, in addition to the availability of Lys and Gln residues, another factor that could potentially affect cross-linking of two different macromolecular protein substrates by transglutaminase is the thermodynamic compatibility of mixing of the protein substrates at the enzyme's active site." (Han and Damodaran, 1996).

The use of transglutaminase began with the manufacture of surimi (fish paste) products in Japan, where the enzyme was able to increase the firmness and elasticity of the surimi gel and also the breaking strength and deformation (Kuraishi et al., 2001).

Transglutaminase is also used in the seafood industry to prevent deteriorative changes such as textural changes in seafood products that are stored frozen (Kuraishi et al., 2001).

In meat, the major substrates of transglutaminase are myosin and actin. Muguruma et al. (1999) indicated that textural characteristics, such as the elasticity and firmness of chicken sausages, could be improved by the addition of transglutaminase. This improvement can be explained by the formation of a protein network in the presence of transglutaminase.

Another meat application is the use of transglutaminase to produce healthy products such as sausages that have reduced salt or phosphate levels. The addition of the enzyme can maintain physical characteristics such as the water-holding capacity, binding, consistency and overall texture when other ingredients have been reduced. Studies in this area showed that the breaking strength of low salt sausages was decreased by 20% when the salt content was reduced from 1.7% to 0.4%. However, the addition of transglutaminase improved the texture and restored the breaking strength, compared with the control sample.

One of the most important uses of transglutaminase in the meat industry is in restructured meat, where small meat pieces can be bound together with the enzyme,

giving products with an enhanced market value as a result, because they have superior slicing, freezing and cooking properties, without affecting the taste and flavor, compared with traditional heat-restructured products (Kuraishi *et al.*, 2001).

Transglutaminase can be used in other systems, such as milk protein, soybean or gelatin, to improve their physical properties and even to expand their possible uses, by improving product hardness and elasticity, binding strength, viscosity, thermo-stability or emulsion stability. Transglutaminase can also be used to reduce or replace the thickener or stabilizer content of a product. As only a small amount of transglutaminase can have satisfactory effects, it does not have unfavorable repercussions on the flavor or color of foods. Furthermore, at the same time, transglutaminase can also be useful for creating new products, for instance, new gel-form foods, such as aspic, that do not melt on cooking. It is also possible to produce fresh restructured meat that is neither frozen nor precooked and will be attractive to the consumer. Making uniform meat products of various sizes or shapes by using transglutaminase leads to effective utilization and added-value enhancement of materials (Sakamoto et al., 1994).

Babin and Dickinson (2001) studied the influence of transglutaminase treatment on the thermo-reversible gelation of gelatin.

A pre-treatment (15 min at 50°C) with transglutaminase, followed by enzyme inactivation (10 min at 70°C) prior to the initiation of cold-set gelation, leads to an increase in the gelation time (by 5 min) and a reduction in the measured time-dependent modulus following gelation. This could be because adding intra- and intermolecular covalent cross-links before gelation makes subsequent interactions between molecules on cooling more difficult and hence influences the setting properties of the gel solutions. However, if transglutaminase (0.015 wt%) is introduced into a gelatin solution at 40°C immediately before the cooling process, the gel strength increases compared with the untreated system (Babin and Dickinson, 2001).

This indicates that, when used with gelatin, transglutaminase may have a positive or negative effect on the gel strength of gelatin depending on whether the covalent cross-linking occurs before or after the formation of hydrogen-bonded triple-helix junction zones during cooling (below 35°C).

Ikura et al. (1980) indicated that transglutaminase gelation depended on the protein concentration and that the substrate concentration had to be higher than 2%, to be able

to form hydro gels with improved gel strength compared with the control. The formation of a hydro gel resulted from covalent cross-links involving ε-(γ-glutamyl) lysine bonding. It was stable to heat treatment up to 100°C, maintaining the original gel state (Arai and Fujimaki, 1991).

In particular, a hydro gel produced from α_{s1}-casein with transglutaminase had an increased storage modulus for a while but eventually showed entropic elasticity (Arai and Fujimaki, 1991).

The above studies indicate that transglutaminase can be used in a wide range of foods. Thus, it was decided that transglutaminase should be evaluated as a potential ingredient in a number of dairy products.

2.3.1 Transglutaminase and Milk Proteins

A large number of investigations on the treatment of milk proteins with transglutaminase have been carried out, showing the effects not only on the gelling properties but also on the emulsifying properties of milk proteins. Use of the enzyme during the processing of emulsion gel products offers new opportunities for developing improved textures in milk-protein-based spreads, desserts and dressings.

For the successful application of transglutaminase in milk products, it is essential that we ascertain which proteins in milk are suitable for the various transglutaminase-mediated reactions. It is important that we know whether or not all the milk proteins are good substrates and whether some may be better than others. Several investigations have been carried out on milk proteins using transglutaminase and have shown that casein is a very good substrate for the enzyme, whereas the globular whey proteins are poor substrates (Kuraishi *et al.*, 2001).

Studies using the various caseins indicated that α-casein and β-casein had good reactivity, but that κ-casein had much lower reactivity.

The reaction with each casein component was followed by estimating the ammonia released by the protein, as shown in Table I (Ikura et al., 1980).

At present, the reasons for the poor reactivity of κ-casein are not clear. The numbers of glutamine and lysine residues in the κ-casein molecule are comparable with those in α-casein and β-casein; thus, the concentrations of these residues in the reaction of each casein component are at the same level under experimental conditions. Therefore, it would appear that the substrate effectiveness of the peptide-bound glutamine residue

depends not on the structure of the polypeptide containing the glutamine residue but on its primary conformation (Ikura et al., 1980). Figure 5 shows the formation of oligomers of α- and β-caseins after the first 30 min: by 120 min, all monomers and oligomers had been converted to polymers. With κ-casein, only about 60% of the monomers had been converted into oligomers and polymers after 120 min. When the enzyme concentration was increased, all the κ-casein was converted to polymer. This indicates the formation, by transglutaminase, of intermolecular cross-links for each casein component.

It is thought that α-casein and β-casein have disordered structures, but that κ-casein has a fairly ordered structure. It is possible that most of the glutamine and lysine residues of κ-casein are buried inside the molecule and thus is inaccessible to transglutaminase (Ikura et al., 1980).

Table I: Amount of ammonia released and the decrease in free amino groups during the transglutaminase reaction after 120 min (Ikura *et al.*, 1980)

	Number (mol/mol of casein) of:	
Casein	Ammonia released	Decrease in free amino groups
α_{S1}	3.5	3.7
β	3.8	3.5
κ	1.0	0.6

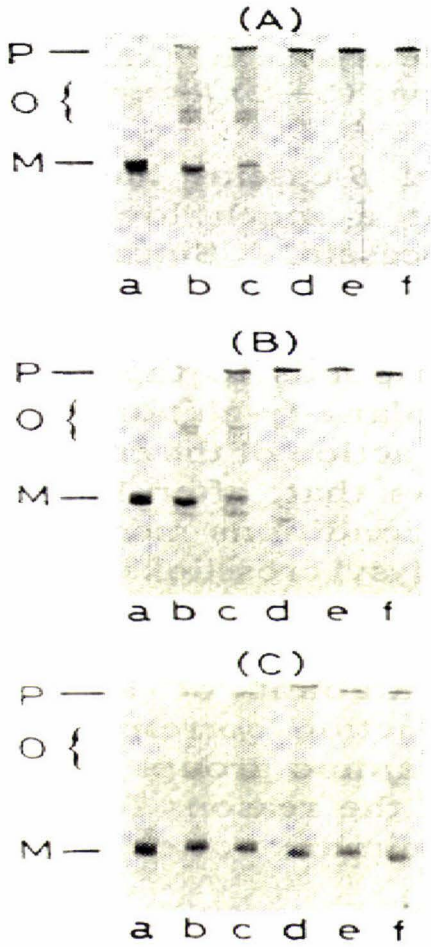

Figure 5: SDS-PAGE patterns of casein components during the transglutaminase reaction.

40 μg of protein was applied to each column. Migration was from top to bottom; M, O and P indicate the positions of bands of monomers, oligomers and polymers.

(A) α_{s1}-Casein; samples on gels a–f, incubated with transglutaminase for 0, 10, 20, 30, 60 and 120 min, respectively. (B) β-Casein: a–f, incubated for 0, 4, 10, 20, 60 and 120 min, respectively. (C) κ-Casein: a–f, incubated for 0, 10, 30, 50, 90 and 120 min, respectively. (From Ikura et al., 1980.)

Tanimoto and Kinsella (1988) studied the effect of time on the cross-linking of milk proteins by transglutaminase (Table II). They showed that transglutaminase activity proceeded linearly with time up to 60 min, and that the rates of activity increased with substrate β-lactoglobulin concentration. The extent of cross-linking of α-casein was greater than that of β-lactoglobulin even though both proteins had comparable molecular weights and similar lysine contents. There were slight differences in glutamine residues with 9 in β-lactoglobulin and 14 in the caseins. These authors speculated that, in spite of the random structure of β-lactoglobulin, easier enzyme access to the reactive groups in the caseins was responsible for the different cross-linking rates.

Another important point is that the rate of cross-linking by transglutaminase is dependent on the macromolecular structure of each protein substrate. Reactive glutamine residues are in the flexible regions of the polypeptide chain or in regions with reverse flexibility and, as a result, the caseins are good substrates. In contrast, it has been widely reported that globular food proteins such as ovalbumin and β-lactoglobulin are not attacked by transglutaminase in their native states (Faergemand, 1997).

The susceptibility of globular proteins to transglutaminase-induced cross-linking may be increased in several ways: by chemical modification, by disruption of intermolecular disulphide bonds, by conversion into the molten globule state or by adsorption at the oil–water interface. Other factors affecting the transglutaminase reaction rate are temperature, pH and calcium ion content (Faergemand, 1997).

At an oil–water interface, transglutaminase could cross-link β-lactoglobulin without any chemical unfolding. The interfacial dilatational elasticity of caseins and β-lactoglobulin at the oil–water interface increased significantly after the cross-linking.

Extensive transglutaminase-induced cross-linking of milk proteins led to greater stability of oil-in-water emulsions and improved creaming of the proteins (Faergemand, 1997).

Table II: Rate of cross-linking of β-lactoglobulin and α_s-casein by transglutaminase (Tanimoto and Kinsella. 1988)

	Reaction time, min			
	15	**30**	**60**	**120**
β-Lactoglobulin				
Percent decrease	6.6	10.0	21.0	28.0
Content. M^3	0.5	0.8	1.7	1.8
Mol/mol protein	1.0	1.5	3.1	3.3
α-Casein				
Percent decrease	12.0	16.0	30.0	34.0
Content. M^3	0.7	0.9	1.8	2.0
Decrease in 10^3 M. mol/mol protein	1.7	2.2	4.3	4.8

1 mg/mL of protein and an enzyme concentration 20 μg of 0.05 units/mL were used.

2.3.1.1 Cross-Linking of Caseins with Transglutaminase

Few studies have been carried out on the cross-linking of the caseins in milk with transglutaminase. However, most of these studies indicate that the cross-link and its efficiency depend on whether or not a protein can be utilised as a substrate by transglutaminase and how the protein is influenced by the amino acid sequence around the reactive glutamines (Gorman and Folk, 1980; cited by Kurth and Roger, 1984). Thus the primary structure of a protein is of greater importance in determining its ability to act as a substrate than its absolute lysine and glutamine content.

Studies by Traoré and Meunier (1991) on the cross-linking of caseins by the transglutaminase Human Placental factor XIIIa (FXIIIa) indicated that whole caseins cross-linked, leading to a species of high molecular weight. The SDS-PAGE results from that research showed the patterns of the products of the cross-linking of α-, β- and κ-caseins, respectively. All of these proteins could be polymerized by transglutaminase, whatever the procedure of activation used (thrombin or calcium).

β-Casein and κ-casein were more susceptible to FXIIIa than α-casein at low concentrations of FXIIIa; all the proteins completely polymerised at a higher FXIIIa concentration. This could have been because α-casein has a lower reactivity because the glutamine and lysine residues are buried inside the polymer (Traoré' and Meunier, 1991).

Normally, whey proteins have to be heated to force them to unfold and expose their reactive groups before they can be successfully cross-linked with FXIIIa. However, no such treatment was necessary for α-casein, even though the heat treatment must have exposed the glutamine and lysine residues of α-casein (Traoré and Meunier, 1991). According to these authors, heat treatment of a protein can expose hydrophobic bonds deep within the tertiary protein structure and these can participate with similar groups on other molecules, with the result that protein aggregation can occur. Heat appeared to have no synergistic effect on the polymerisation of β- and κ-casein.

Studies by Lorenzen (2000) on the properties of transglutaminase-treated milk proteins indicated that the hydration ability of α_{s1}-casein was increased by the cross-linking reaction. The solubility and emulsifying properties of α_{s1}-casein in the pH range 4–6 were also improved (Lorenzen, 2000).

α_{s1}-Casein in milk powder, polymerised by transglutaminase, showed an increase in the glass transition temperature, whereas κ-casein was hardly influenced by a transglutaminase treatment (Lorenzen, 2000).

Kuraishi et al. (2001) indicated that β-casein was a better substrate for transglutaminase. As a consequence, it has been used to study the specificity of the transglutaminase-catalysed modification of glutamine residues in proteins.

Finally, β-casein and transglutaminase have been used to study aspects of glucoprotein structure in lectin–sugar interactions using rheological measurements (Yan and Wold, 1984; cited by Christensen, 1996).

Han and Damodaran (1996) examined the effect of treating a mixed protein system (β-casein and β-lactoglobulin) with transglutaminase. They found that only β-casein was polymerized by transglutaminase and that β-lactoglobulin was left untouched. They concluded either that β-casein was a better substrate for the enzyme or that β-casein somehow interfered in the interaction of β-lactoglobulin with transglutaminase. However, a system containing both α- and β-casein showed that transglutaminase polymerized the two proteins.

Studies on caseins in an emulsion system treated with transglutaminase indicated that at low protein concentration (0.2 wt%) the proteins cross-linked prior to homogenisation and as a consequence affected the droplet size. Moreover, because the proteins had partially aggregated, they were not able to prevent coalescence and bridging flocculation. However, at higher protein concentrations, there was enough protein to cover the droplet surface in its aggregated state, so that there was no reduction in emulsifying capacity (Faergemand et al., 1999).

The results also indicated that the rate of cross-linking of the casein-coated droplets was much faster than that of the β-lactoglobulin-coated droplets; this is consistent with the much greater susceptibility of casein to the enzyme, reported previously (Dickinson and Yamamoto, 1996).

2.3.1.2 Cross-Linking of Whey Proteins with Transglutaminase

Milk proteins need to be unfolded prior to being treated with transglutaminase.

Singh (1991) mentioned studies by Tanimoto and Kinsella (1988), in which native β-lactoglobulin (at 10% w/v) formed hard, clear gels upon heating at 95°C for 30 min. However, the proteins cross-linked by transglutaminase showed a loose, soft coagulum

under similar conditions. This indicated that the formation of intramolecular cross-links could impede the thermally induced unfolding of protein molecules, which, as a consequence, limited gel formation.

Transglutaminase has the ability to cross-link pure and mixed whey protein preparations. Aboumahmoud and Savello (1990) studied purified α-lactalbumin, β-lactoglobulin, whey powder (12% protein) and modified whey powder (35% protein) with transglutaminase at different pHs, incubating at 37°C for 4 h. They showed that all the protein substrates were cross-linked by transglutaminase.

The disappearance of protein bands, the appearance of new protein bands and the accumulation of immobile protein polymers at the SDS-PAGE gel origin demonstrated the extent of the cross-linking. Transglutaminase had the ability to cross-link both globular and non-globular milk proteins (Aboumahmoud and Savello, 1990). These researchers also analysed the transglutaminase activity at pH 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 using whey proteins. They showed that the enzyme was active over a wide pH range. Whey powder underwent transglutaminase protein cross-linking between pH 6.5 and pH 8.5. The highest production of immobile cross-linked protein polymer was observed at pH 7.5 and the lowest production was at pH 8.5. At pH 9.0, no cross-linking was observed (Aboumahmoud and Savello, 1990).

Transglutaminase cross-linking of α-lactalbumin, β-lactoglobulin and a 1:1 mixture of α-lactalbumin and β-lactoglobulin showed that a mixture produced similar protein polymer bands to the cross-linked pure fractions.

The mixed protein and modified whey powder proteins exhibited more cross-linking and polymerisation than the pure fractions.

The demonstrated cross-linking increased with increasing protein concentration (1 and 5%). Between these concentrations, there is the option of using proportionately less transglutaminase or increased protein concentration to demonstrate a similar degree of dairy protein cross-linking by transglutaminase (Aboumahmoud and Savello, 1990).

Incubation time was investigated using α-lactalbumin, β-lactoglobulin and modified whey powder protein as substrates. This study showed that, for α-lactalbumin and β-lactoglobulin, protein polymer formation generally increased during the 4 h incubation. Continued incubation after 4 h did not increase the quantity of cross-linked protein.

Aboumahmoud and Savello (1990) found that dithiothreitol (DTT) was required to cross-link whey proteins with (guinea pig liver) transglutaminase. They also showed

that the film-forming ability of reduced whey proteins cross-linked with transglutaminase could be improved by adding glycerol.

A study of the enzymatic cross-linking of whey proteins by a calcium-independent microbial transglutaminase from *Streptomyces lydicus*, carried out by Faergemand et al. (1997), showed that α-lactalbumin was mostly cross-linked by transglutaminase and that β-lactoglobulin was apparently only cross-linked slightly by transglutaminase, even though the concentration of the protein in the samples containing transglutaminase was not significantly lower than in the control samples.

These results suggest that α-lactalbumin possesses both glutamine and lysine residues that are available for cross-linking with microbial transglutaminase, whereas β-lactoglobulin must lack reactive glutamine or lysine.

2.4 Applications of Transglutaminase in the Dairy Industry

Transglutaminase can be used in model systems using substrates such as whey protein, casein and fractions of these proteins. It can be used to modify the functional properties, to improve the hydration ability, gel formation, rheological and emulsifying properties as well as the heat stability of proteins in the dairy industry. In fermented milk products, in particular, enzymatic cross-linking could be a very important technological application (Table III) (Lorenzen and Schlimme, 1997).

Control of the texture of the milk protein gel may be achieved by combining the transglutaminase treatment with thermal treatment (whey proteins) or acidification (caseins). Physical network gels are typically susceptible to serum separation (syneresis) as a result of post-gelation structural rearrangements. In milk protein gels formed by heating, renneting, pH change or even high pressure treatment, the presence of additional enzyme-induced covalent cross-links could be beneficial in inhibiting structural reorganization and associated syneresis during long term storage (Dickinson, 1998).

Nonaka et al. (1992) studied the characteristics of sodium caseinate gels with transglutaminase, and found that the control, which was incubated with inactivated enzyme, did not form rigid gels. The gels would not stand and maintain their cylindrical shape when the casing tube was removed. The apparent features of transglutaminase-treated sodium caseinate solutions (water suspensions and emulsions) depended greatly on the enzyme concentration and the sodium caseinate concentration.

Texturometer analysis results indicated that there was a substantial difference between the cohesiveness of the gel made with low enzyme concentration and that of the gel made with high enzyme concentration. The maximum hardness was obtained with an enzyme concentration of 30 units/g protein in both the solution series and the emulsion series.

Table III: Potential use of transglutaminase in the processing of milk proteins and products (from Lorenzen and Schlimme, 1997)

(↑) = Increasing, (↓) = Decreasing

Product	**Expected aim of enzymatic modification**
Yogurt, fresh cheese	Gel strength (↑), syneresis (↓)
Fresh and ripened cheese	Product yield (↑), syneresis (↓)
Ice cream	Water binding (↑), gelation properties (↑)
Caseinates	Gelation and emulsification properties (↑), viscosity (↑)
Desserts	Gelled milk concentrates as ingredients
Whipping cream	Improvement of physical properties
Microparticulated proteins	Fat replacer and substitutes
Novel milk products	For example, spreads, low calorie foods
Proteolysates	Covalent incorporation of lysylpeptides
Whey proteins	Formation of packaging film
α_{s1}-Casein	Edible films and coatings, medical polymers (e.g. artificial skin), enzyme immobilizing

The gels made using transglutaminase were divided into two types: those that were difficult to break by axial compression, namely the gel incubated at an enzyme concentration of 10 units/g protein; those that were more fragile under axial compression, namely the gel incubated at an enzyme concentration 50 or 100 units/g protein (Nonaka et al., 1992).

Studies on gelation in skim milk indicated that, at two solids content levels, 43.1% (w/w) and 50.3% (w/w), respectively, and two protein concentrations, 15% (w/w) and 17.5% (w/w), respectively, the breaking strength and the hardness of the gel were increased at the higher enzyme concentration, whereas there was almost no change at the lower concentration. The strain and the cohesiveness were not affected as much by the increased enzyme concentration (Nonaka et al., 1992). This shows the capability of transglutaminase to act as a unique agent for preparing gels with many different physical characteristics from commercial milk products such as sodium caseinate and skim milk (Nonaka et al., 1992).

Investigations into soy protein isolate (SPI), caseinate, gelatin, egg yolk and egg white with the addition of transglutaminase showed that the breaking strength of caseinate and gelatin increased sharply with increased transglutaminase concentration. The maximum breaking strengths at a given enzyme concentration differed markedly among the protein substrates. The optimum strengths were obtained with an enzyme concentration of 40–50 units/g protein in SPI, 15-units/g protein in caseinate, 30 units/g protein in gelatin and egg yolk, and 10–30 units/g protein in egg white gels. In the caseinate, gelatin and egg yolk gels, a decrease in breaking strength was observed above 15, 30 and 30 units/g protein, respectively. Those gels at high enzyme concentration were not only soft but also fragile. A hypothesis for this could be that excessive formation of glutamyl-lysine (GL) cross-links would inhibit uniform development of the thermally induced protein network (Sakamoto et al., 1994).

Studies by Lorenzen and Schlimme (1997) on the enzymatic cross-linking of selected milk proteins indicated that sodium caseinate could be used as a model substrate for an open protein chain and that whey protein could be used as a model of a globular protein, when using transglutaminase.

The results obtained showed that cross-linking with transglutaminase, in particular of the casein molecules, takes place at fast reaction rates. In addition, the open chain tertiary structure of the caseins causes a higher degree of cross-linking compared with the globular structure of the whey proteins. After incubation for 2–4 h, the casein

molecules are almost completely cross-linked to form high molecular weight aggregates, although the contents of soluble amino-N-, regarded as a measure of the number of free amino groups, decrease by only approximately 5% compared with the substrate. Aggregates of a lower molar mass are formed only as intermediates. The protein sodium caseinate is used in cross-linking meat pieces during the manufacture of frankfurter type sausages and restructured meat products (Lorenzen and Schlimme, 1997).

2.4.1 Use of Transglutaminase in Yogurt

Acid milk gels, e.g. yogurt, are commercially important milk gels. There are two essential parameters for consumer quality acceptance: consistency, and low syneresis or whey drainage. Introducing new covalent bonds into the gel may change these parameters.

Studies on the production of yogurt and its characteristics indicated that increasing the protein concentration of skim milk from 3.5% to 7.0% by means of mono-filtration led to firmer gels, increased viscosity and increased serum-holding capacity. These structural properties indicated that, the more protein there is in the system, the finer is the gel network (Schkoda et al., 2001).

However, studies by Lorenzen and Schlimme (1997), using yogurt and the addition of transglutaminase, indicated that the enrichment of dry matter and/or of the protein content seems to be insufficient to prevent syneresis in yogurt gels, as mentioned above. A potential solution to this problem could be the use of transglutaminase in gels, which, as a result of the protein cross-linkages, could produce a finer-meshed gel network and therefore could be used to replace the addition of milk solids or an increase in the fat content (Faergemand and Qvist, 1997).

Lorenzen and Schlimme (1997), in their study on the addition of transglutaminase to yogurt, also concluded that transglutaminase could reduce syneresis by cross-linking protein chains to stabilize the three-dimensional network of the gel.

In contrast to modified skim milk, the fermentation time of cross-linked milk was longer than that of the control. There were no differences in titratable acidity during the storage of modified or non-modified yogurt products.

Increasing the incubation time of milk led to a decrease in the syneresis of the resulting yogurts and increase in the gel strength (Figure 6-7). The same was true for increasing the protein content by adding sodium caseinate. An enhancement of the dry matter

content with whole milk powder before cross-linking had no visible influence on yogurt stability in relation to the control. In addition, sensory analysis indicated that yogurt products prepared from cross-linked milk were perceived to be creamier than the control products (Lorenzen and Schlimme, 1997). This result is in agreement with findings described by Nielsen (1995). Studies on set style acid skim milk gels by Faergemand and Qvist (1997) indicated that transglutaminase affected the rheological properties of milk gels. The complex modulus (the total stiffness of the gel, arising from the viscous and elastic responses) of transglutaminase-treated gels was up to six times greater than that of the non-transglutaminase-treated gel.

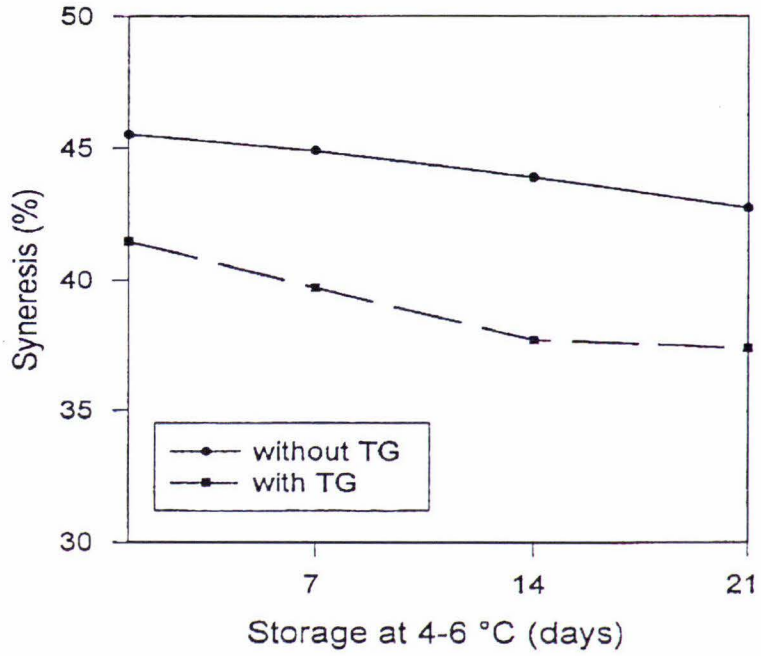

Figure 6: Syneresis of transglutaminase-treated skim milk yogurt in relation to the non-treated product (from Lorenzen and Schlimme, 1997).

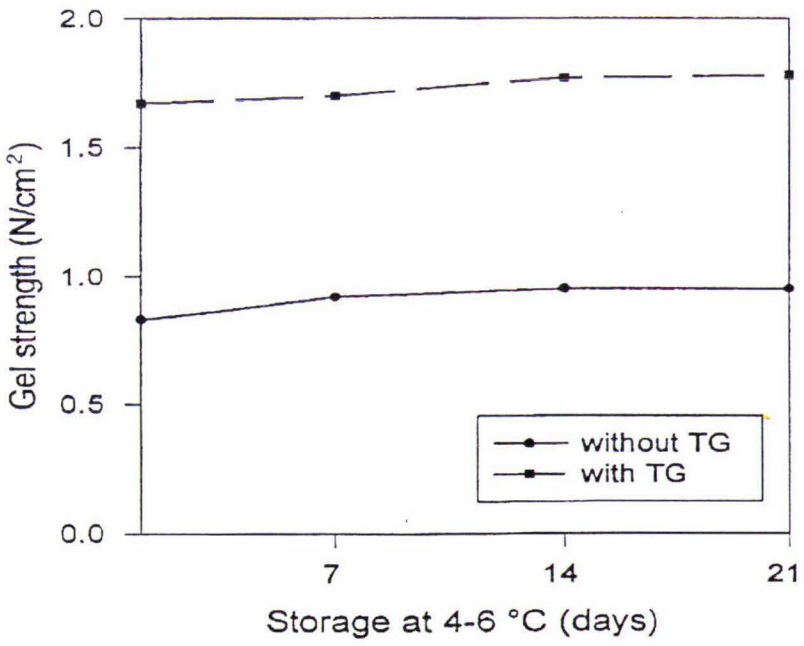

Figure 7: Gel strength of transglutaminase-treated skim milk yogurt in relation to the non-treated product (from Lorenzen and Schlimme, 1997).

2.4.2 Use of Transglutaminase in Cheese

The renneting or clotting ability of milk is a very important property in cheese making. The first step in renneting is the proteolytic attack of rennet on κ-casein. Physical factors, e.g. the temperature and pH of the milk as well as the activity of the enzymes, are important in renneting. The second step in renneting is the destabilisation of the casein micelles (clotting), which depends on the calcium concentration of the milk. Lorenzen and Schlimme (1997) studied the effect of the enzymatic cross-linking by transglutaminase on the renneting ability of skim milk. They found that the renneting ability depended on the temperature of the preheating step and the time of incubation with transglutaminase. A small degree of cross-linking and a slight increase in clotting time were found when milk was heated to 37°C. However, when milk was heated to 75°C, significant increases in cross-linking and clotting time were noted. At 90°C, cross-linking in the milk was inhibited completely. "This inhibition of clotting may be explained by an increasing degree of cross-linking between unfolded globular proteins and micelle bound casein molecules. A kind of "surface-sealing" of the casein micelles may take place that prevents cleavage of κ-casein." (Lorenzen and Schlimme, 1997).

Experiments to assess the effect of the pre-treatment of milk with transglutaminase prior to renneting showed that, when rennet was added after a 10-min incubation with transglutaminase, this led to relatively high clotting times. Simultaneous addition of the two enzymes reduced the time for clotting. The shortest renneting times within this system were found if transglutaminase was added after a 10-min fermentation with rennet. In addition, reduced clotting times were observed when the pH was decreased or when there was an increase in the calcium concentration (Lorenzen and Schlimme, 1997).

Three options proposed by Kuraishi et al. (2001) for the application of transglutaminase in the manufacture of cheese are given below.

Option 1: Adding transglutaminase to milk, heating the milk to pasteurise it and to deactivate the enzyme, and then adding rennet to the milk.

Option 2: Adding rennet to milk and then adding transglutaminase.

Option 3: Adding transglutaminase to milk at the same time as rennet.

The results obtained for Gouda cheese indicated that milk treated with transglutaminase (0.2 units/g protein) at 25°C for 2 h showed an increase in yield of 9.10% compared with the control which showed 8.79% (Kuraishi et al., 2001).

2.4.3 Use of Transglutaminase in Cream

Cream products tend to separate when stored for any length of time, and overcoming this problem is vitally important to the dairy industry. Lorenzen and Schlimme (1997) studied the effect of transglutaminase cross-linking on the physical properties of whipping cream and showed that transglutaminase enhanced the viscosity of protein solutions and reduced time of whipping. Consequently, transglutaminase could be used instead of stabilizers such as carrageenan.

The results from their studies showed an increase in overrun, a slight increase in viscosity, a small decrease in creaming and a reduction in whipping time after cross-linking with transglutaminase (Table IV).

2.4.4 Use of Transglutaminase in Films

Another application of enzyme-cross-linked casein networks is the formation of thin, macroscopic films for use as biodegradable packaging materials or edible coatings for enhancing food quality.

The covalent cross-linking of whey proteins by transglutaminase can be used to produce films. Covalent cross-linking of whey proteins, in the presence of active transglutaminase, produced a polymerised network of whey proteins and resulted in the gelation of α-lactalbumin and β-lactoglobulin at and above 3 and 5% protein concentration, respectively. All the films were transparent (Faergemand et al., 1999).

It was possible to produce peelable whey protein films by the catalytic mediation of transglutaminase with the addition of a plasticiser to the reaction mixture (Mahmoud and Savello, 1993).

The protein polymer network of films resisted solubilization in aqueous buffers at various pH and heat treatments; however, it was protease digestible. Use of a cross-linked whey protein as an edible film or a food-coating material depended on the acidity and the proteolytic activity of the coated surface (Mahmoud and Savello, 1993).

Table IV: Properties of cross-linked whipping cream in relation to the non-treated product (Lorenzen and Schlimme. 1997)

- Slight increased viscosity of the liquid cream
- Slight reduced creaming of the liquid cream
- Reduced time of whipping
- Increased overrun (foam volume) of the whipped cream
- Slight decreased firmness of the whipped cream
- Comparable serum leakage (syneresis) of the whipped cream

2.5 Nutritional Importance of Transglutaminase

The incorporation of amino acids into food proteins has become very important, because of the pressures to feed an increasing population. Resources are limited and therefore the effective utilization of amino acids in food proteins must be improved using new methods.

The enzymatic modification of proteins could be a promising method to improve the functional properties and nutritive value of food proteins.

It has been demonstrated that transglutaminase can be useful for polymerising food proteins, forming intermolecular cross-links. These cross-links could also be useful for the incorporation of amino acids into a range of foods to improve their biological value.

Ikura et al., (1981), in a study on the incorporation of amino acids into food proteins, showed that methionine is a limiting essential amino acid in milk and soybean proteins and that transglutaminase could be used to incorporate it. Their experiments indicated that, after 120 min, the methionine content of α_{s1}-casein was 2.0 times greater than that of its starting material and that of β-casein was 2.4 times greater. The cross-links were detected using SDS-PAGE.

A similar amino acid deficiency is true for wheat gluten, where lysine is a limiting essential amino acid. It was shown by Ikura et al., (1981) that this amino acid could be incorporated into wheat gluten by using transglutaminase.

Hurrell and Carpenter (1977), cited by Nielsen (1995), indicated that, in studies on the digestibility of aspartyl-lysine and glutamine-lysine cross-linkages by heating, the dipeptide appeared to be digestible as total protein.

Studies on caseinate gels produced by cross-linking with guinea pig liver transglutaminase showed that the resulting proteins were highly digestible (Choi et al., 1983; cited by Nielsen, 1995).

The nutritional fortification of proteins with essential amino acids by transglutaminase can be of importance only if it can be demonstrated that the ε-(γ-glutamyl) lysine bond is bioavailable to the body.

Studies by Seguro et al. (1995) indicated that the cross-linked protein could be digested by a peptidase "γ-glutamyl-amine cyclotransferase" (γ-GTP). This enzyme spreads the dipeptide ε-(γ-glutamyl) lysine into lysine and 5 oxo-L-proline.

This suggests that, during gastrointestinal digestion of cross-linked protein, γ-GTP in the intestinal mucosal wall, the protein would be hydrolysed and lysine would be utilised as normal.

Later, Seguro et al. (1996) studied the ε-(γ-glutamyl) lysine moiety in cross-linked casein, and found that casein is an available source of lysine for rats and confirmed the bioavailability of the dipeptide, expressed as protein efficiency ratio (PER) and biological value (BV). The results showed that the PER and BV in rats that were fed with the dipeptide were similar to those in the rats fed with control diets (without glutamyl lysine intake). The results were obtained by high performance liquid chromatography (HPLC) analysis of urine and faeces, indicating negative presence of the dipeptide. Finally, 99% of the ε-(γ-glutamyl) lysine moiety in the cross-linked caseins was consumed and absorbed through the gastrointestinal tract.

2.6 Determination of ε-(γ-Glutamyl) Lysine

The ε-(γ-glutamyl) lysine isopeptide bond, which is formed by cross-linked proteins with transglutaminase, can be measured by using phenylisothiocyanate derivatisation and HPLC separation.

The HPLC procedure is based on the separation of the dipeptide on a cation exchange resin and a silica HPLC column, and pre-column derivatisation with phenylisothiocyanate, using a reversed-phase HPLC separation on a C18 column.

The derivatised isopeptide gives a linear concentration response relationship, with a detection limit of 10 mol/mg of protein. The combination of the preliminary separation steps and the sensitive detection system permits the determination of the ε-(γ-glutamyl) lysine cross-link in complex biological systems including total tissue homogenates (Tarasa and Fesus, 1990).

A similar method for ε-(γ-glutamyl) lysine cross-link determination was used by Sakamoto et al. (1995) in a study of 96 different foods.

Christensen et al. (1996) suggested radio labelling of reactive amines to detect transglutaminase-reactive glutamines.

An indirect method could be to measure the amount of ammonia released by the cross-linking reaction (Ikura et al., 1980), but this could result in an overestimation because other deamination reactions in the system could also produce ammonia. Therefore,

Motoki and Nio (1983) suggested that SDS-PAGE could be another way to measure the degree of polymerisation by weight distribution of the proteins.

2.7 Conclusions

The literature review indicates that the use of transglutaminase as an ingredient for food processing is increasing worldwide because of its great potential to improve the firmness, elasticity, viscosity, heat stability and water-holding capacity of prepared foods. It has already been used in seafood, surimi and meat products, and now in dairy products.

It has been suggested that transglutaminase can convert a liquid part into a viscoelastic emulsion gel, increasing the thermal stability and water holding capacity. It can also improve the surface activity of a protein by attaching non-polar groups to the side chains of certain amino acids (Dickinson and McClements, 1995).

Studies done on the effect of transglutaminase with milk proteins have indicated that the ability of milk proteins to become trasglutaminase's substrate will depend on the amino acid sequence and lysine, glutamine content (Kurth and Roger, 1984). Most studies indicated that casein were better substrates than whey protein, this because its amino acids require of spatial structure. However, Tanimoto and Kinsella (1988) demonstrated that when whey proteins were heated, the protein-enzyme interaction increased, and this because amino acid exposure.

As a result of enzymatic casein cross-linkage, the food industries could satisfactory improves the functional properties of milk products. And it could be a valuable tool for controlling physical properties that may be of importance for the stability of a product during its shelf life.

In this research, a limited number of functional properties were investigated in milk proteins and milk products, but clearly more research could reveal if cross-linking will produce beneficial effects on other functional properties.

CHAPTER III

5.6 MATERIALS AND METHODS

5.6 MATERIALS

3.1.1 Trim™ Milk and Full Fat Milk

Trim™ milk and Full Fat milk were purchased from local supermarkets for each trial, during March and May 2001 and immediately stored at 5°C until used.

3.1.2 Transglutaminase

Transglutaminase (glutaminyl–peptide γ-glutamyl transferase), derived from a microbial source (*Streptoverticillium* sp.), was supplied as a powder consisting of 10% transglutaminase and 90% maltodextrin (bulking agent) by Ajinomoto Co., Inc., Japan.

3.1.3 Milk Protein Concentrates

ALANATE 185: sodium caseinate (T1018 from NZMP)

ALACEN 132: whey protein concentrate (WPC – U 3007 from NZMP)

ALAPRO 4850: milk protein concentrate (MPC85 IL 30 from NZMP)

ALAPLEX 1150: milk protein isolate (146 – U 2004 from NZMP), which contains caseinate and denatured whey protein (90% protein).

3.1.4 Yoghurt Starter: YC 180, freeze-dried *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, supplied by the manufacturer Christian Hansen, Hørsholm, Denmark.

3.1.5 Petit Suisse Starter: R 704, freeze-dried *Lactococcus lactis* subsp. *Lactis* and *Lactococcus lactis* subsp. *Cremoris*, supplied by the manufacturer Christian Hansen, Hørsholm, Denmark.

3.1.6 Homogenizer

APV Rannie, Albertlund, Denmark

Model LAB, and type 12.50H. Pressures used: 150/50 bars.

Table V: Composition of liquid milk (Composition of New Zealand Dairy Foods)

Per 100 g Product

PROXIMATES	Unit	Trim milk	Full fat milk
Water	g	88.8	87.7
Energy	KJ	170	280
	Kcal	42	67
Protein	g	4.3	3.28
Total fat	g	0.42	4
Available carbohydrate	g	5.6	4.7
Lactose	g	5.6	4.7
Sucrose	g	NA	NA
Ash	g	0.92	0.71
Cholesterol	mg	3.5	11.7
MINERALS			
Calcium	mg	146	114
Phosphorus	mg	113	87
Sodium	mg	51	39
Potassium	mg	203	150
Magnesium	mg	11.8	9.3
Sulphur	mg	39	28
Iron	mg	0.021	0.027
Zinc	mg	0.46	0.37
Copper	mg	2.9	3
Manganese	mg	4	5

3.2 METHODS

3.2.1 Determination of Transglutaminase Activity

Transglutaminase activity was determined by the hydroxamate procedure with CBZ-L-glutaminyl-glycine as substrate (Harnett, 2002). The enzyme activity unit was defined as one unit (U) causing the formation of 1 μM of hydroxamate per minute at 37°C. The specific activity of the enzyme preparation was 1095 U/g.

3.2.2 Transglutaminase Preparation

Sequential dilutions of a solution of the enzyme were prepared. Enzyme (10.000 ± 0.0005 g) was weighed into a tared beaker, and then transferred to a 200 Ml volumetric flask. The flask was made up to the mark using reverse osmosis (RO) water. The enzyme concentration/activity of this solution was 40–50 U/Ml.

This initial solution was then further diluted by pipetting 10 Ml into a 100 Ml volumetric flask, and then making up to the mark with RO water (enzyme concentration/activity 4–5 U/Ml). The base solution was stored at 5°C for no longer than 1 month.

3.2.3 Sample Preparation to Determine the Effect of Transglutaminase on Milk proteins

Full Fat and Trim™ milk were used in the experiments to determine what effect transglutaminase would have on the milk proteins. Some of the Full Fat milk was homogenized at 150/50 bars (60°C) to see what effect this processing step would have on transglutaminase mediated cross-linking of the proteins in this type of milk.

Then all milk samples (Trim™ and Full Fat) were submitted to one of two treatments: an unheated control or heated to 90°C for 10 min. Then a known weight of milk (300 Ml) was placed in a beaker and into a water bath at either 37°C or 55°C (enzyme incubation temperatures for this research). Transglutaminase (0, 1, 5, 10 and 100 U/Ml) was added to the respective milk samples, and stirred with a Heidolph stirrer for 2 hrs. The enzyme was then inactivated by heating the milk sample to 90°C for 2 minutes in a microwave, followed by storage at 5°C.

3.2.4 Sample Preparation to Determine the Effect of Transglutaminase on Milk proteins and Milk Protein Concentrates

Four 1.5 L batches of both Trim^{TM} and Full Fat milk were used. Each batch was mixed with either ALANATE 185 (sodium caseinate (NaCN)) 0.5% w/w, or ALACEN 132 0.625% w/w, or MPC 0.5% w/w or TMP 0.5% w/w respectively, at 60°C for 30 min to dissolve the powder.

Once a milk solution had been obtained, each batch was homogenised at 150/50 bars (60°C) and then heated at 90°C for 10 min to denature (i.e. unfold) the whey proteins. The solution was then cooled to either 37°C or 55°C, at which stage one of the following concentrations (0, 1, 5, 10 and 100 U/Ml) of transglutaminase was added to the milk samples. The milk solutions were stirred continuously for 2 hours with a Heidolph stirrer. The enzyme was then inactivated by heating the solution to 90°C for 2 min in a microwave. All samples were then cooled and stored at 5°C until assessed.

3.2.5 Total Solids

Total solids were measured in duplicate by weighing a 2 g sample into a pre-weighed moisture dish, which was left to dry in a vacuum muffle at 100°C for 20 min (Gallenkamp, made in England) Appendix A1, 2,3 and 4.

3.2.6 Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

When a fresh milk sample is analysed using native PAGE, the proteins are separated into bands of native monomeric proteins. If the milk sample is heated prior to analysis, some of the proteins will denaturate and form non-covalent and/or covalent cross-linkage aggregates. If this sample is analysed using native PAGE, only the native monomeric proteins will be separated in the gel. All the aggregated proteins will remain on top of the stacking gel. If the same sample is analysed using SDS-PAGE, the non-covalent linked proteins will dissociated and run as bands of SDS-Monomeric proteins (Gezimati et al., 1996; Havea et al., 1998), while the covalently linked aggregates remain on top of the stacking gel. If the milk sample is analysed using SDS-reduced PAGE, all aggregates (including covalent linked aggregates) will resolve into monomeric proteins and get separated in the gel.

When a milk sample is treated with transglutaminase, covalently linked aggregates will be formed. When this sample is run alongside an untreated control sample on an SDS-PAGE the amount of cross-linked proteins, due to the action of transglutaminase can be estimated on the basis of the differences between the densitometry intensities of the protein bands. The intensity of the bands in the non-treated sample could be considered 100% monomeric. When treated sample is run on the same gel, the cross-linked proteins will remain on top of the stacking gel, resulting in a reduction of the intensities of the separated protein bands. This reduction can be measured and used as an indication of the activity of the transglutaminase (Gel Electrophoresis, Food Science department, FRC) Appendix B.

3.2.7 Sample Preparation for Acid Milk Gels

A solution of milk was prepared by slowly dissolving 14% whole milk powder (WMP) in tap water at approximately 40°C. The prepared milk solution contained 3.5% fat. The milk was then warmed up to 60°C, homogenised at 150/50 bar and heated for 10 min at 90°C (the milk was heat treated to unfold the proteins in the system). The heated milk was then cooled to 37°C and incubated with 0, 10, 50, 100 or 200 U of transglutaminase/Ml of milk. The transglutaminase-treated milk was then given one of two treatments (cases) (see Figure 24). In Case 1 the milk was treated with transglutaminase-treated (for 2 hours at 37°C and then heated to 90°C for 2 min to inactivate the enzyme and then cooled to 40°C. The starter was then added and the milk was then put into a 100 Ml bottle and incubated at 40°C for 16 hours and then stored at 5°C until analysed. In Case 2 the milk was treated with transglutaminase in the same way as for case 1. Starter was added at the same time as the transglutaminase. At the end of the of the 2 hour incubation period the milk was place in a 100 Ml bottle and stored for 16 hours at 40°C and then cooled to 5°C until analysed.

3.2.8 Sample Preparation for Petit Suisse

Petit Suisse was prepared in the same way as the yoghurt, with the addition of MPC56, CP70, and Alacen132 using *Lactococcus lactis* subsp. *Lactis* and *Lactococcus lactis* subsp. *Cremoris* as starters.

3.3 Measurements on Acid Milk Gels

Gel strength. syneresis and Ph were measured. Syneresis and gel strength were also measured at day 1 and day 15.

3.3.1 Texture Profile

The product gel strength was measured in a cooled room at 6°C. A Universal TA–XT2 Texture Analyser with a real time graphics and data acquisition software package (XTRA Dimension) from Stable Micro System. Haselmare. England. was used to obtain texture profiles of the gels.

A 10 mm diameter probe was pushed into the samples (5°C) at a constant rate (1 mm/s) for a distance of 20 mm. and was then withdrawn at the same rate. The response was measured as force vs. time. The force required penetrating the product. the force generated by penetration to the maximum depth and the positive area under the force/time curve were measured. Every run was performed in triplicate and the gel strength was reported as (g).

3.3.2 Free Whey

The yoghurt samples were held at 5°C overnight. After weighing the yoghurt and beaker. any free whey on the surface of the yoghurt was carefully poured off and weighed. Free whey, as a percentage of the total yoghurt. was calculated as follows:

$$\frac{\text{Free whey (g)}}{\text{(Yoghurt + pottle) (g)} - \text{pottle (g)}} * 100$$

3.3.3 Ph

The Ph of the product was measured using an Orion Model 520A Ph meter. The Ph meter was calibrated against Ph 7.0 and Ph 4.0 buffers before use.

3.4 Statistical Analysis

The data was analyzed using the SAS (1999-2001) Release 8.2 by SAS Institute Inc., Cary. NC, USA)

Analysis of variance was performed on the data to determine the significance of the effects of transglutaminase treatment. enzyme concentration and storage on the physical properties of the various milk systems (Appendix F).

CHAPTER IV

4.0 EFFECT OF TRANSGLUTAMINASE ON MILK PROTEINS

4.1 INTRODUCTION

Various authors (Kinsella and Whitehead, 1989; cited by Dickinson and McClements, 1995, also Kuraishi et al., 2001) have studied enzymatic cross-linking using transglutaminase in different food systems.

Transglutaminase catalyses acyl transfer reactions between peptide glutamine residues as donors and various primary amino acids such as lysine (Faergemand, 1998). This enzyme can form intra- or intermolecular ε-(γ-glutamyl) lysine cross-links.

It has been reported by Ikura *et al.* (1980) that caseins are very good substrates for transglutaminase, and that the efficiency of casein as a substrate is due to its similarity in structure to fibrinogen (Kurth and Rogers, 1984).

The different casein proteins react differently to transglutaminase. β-Casein shows higher reactivity than α-casein. This difference is ascribed not only to the total number of glutamine and lysine residues but also to the enzyme specificity (Traoré and Meunier, 1991). Studies done on the cross-linking of caseins by Human Placental Factor XIIIa showed that α-casein was less reactive than β-casein and κ-casein, and this could be explained by a combination of factors: the absolute lysine and glutamine content and the specificity towards accessible glutamine residues. Native α-casein is known to form a soluble polymer by self-association (Payens and Vreeman, 1982; cited by Traoré and Meunier, 1991). This self-association could result in some reactive glutamine and lysine residues being buried inside the polymer, which could in part explain why α-casein is less reactive to transglutaminase than β-casein.

Dickinson, (1997) indicated that globular proteins such as ovalbumin and β-lactoglobulin are not attacked by transglutaminase in their native state. To increase the susceptibility of globular proteins to transglutaminase cross-linkage, several methods such as chemical modification, disruption of intermolecular disulphide bonds, conversion into the molten globule state and adsorption at the oil–water

interface have been investigated and shown to have a positive effect on transglutaminase-mediated cross-linking of the proteins.

Traoré and Meunier (1992) have shown that whey proteins cross-link incompletely with transglutaminase unless they are unfolded using chemicals such as the reducing agent dithiothreitol (DTT).

A heat denaturation of α-lactalbumin and β-lactoglobulin before the addition of transglutaminase was also investigated and the authors concluded that the heat treatment did not replace the reducing agent in the polymerisation of α-lactalbumin but it helped to unfold the proteins.

The objective of this part of the research was to evaluate how transglutaminase of differing concentrations and subjected to two thermal conditions affected the cross-linking between a number of milk proteins.

4.2 RESULTS

4.2.1 Effect of Heat

The aim of this experiment was to determine what effect a preheat treatment (90°C for 10min) compared to a control would have on milk samples incubated with transglutaminase at either 37°C or 55°C

(Section 3.1.1 and 3.1.2)

4.2.1.1 Effect of heat treatment on Trim™ milk samples incubated with Transglutaminase

In this experiment Trim™ milk was either unheated prior to transglutaminase addition (1, 5, 10, 100 U/Ml) or heated to 90°C for 10 minutes and then cooled to 37°C and 55°C before adding transglutaminase. The two lots of transglutaminase treated Trim™ milk were then incubated at 37°C for two hours. Sample preparation is explained in section 3.2.3. Figure 8 shows the SDS-PAGE gel of Trim™ milk treated with transglutaminase. The gel was divided into two sections. The first six lanes show the unheated Trim™ milk samples. Lanes 1 and 2 are the control samples with no added enzyme. Lanes 7-12 are the heated samples with lanes 11 and 12 as the control samples. The unheated samples incubated with transglutaminase (lanes 3, 4, 5 and 6) showed noticeable reduction in caseins compared to the control samples (lane 1–2). The reduction in casein content was directly proportional to the amount of added transglutaminase with the 100 U/mL

sample (lane 6) showing the largest removal of the caseins, i.e., almost half the size of the band for α and β-casein, while κ-casein was reduced nearly completely compared to the control samples. Similar results pertained for the whey proteins. It is important to highlight the complete disappearance of β-lactoglobulin in the gel when treated with transglutaminase. While α-Lactalbumin also showed monomeric reduction when compared with the control, and its reduction was affected by the transglutaminase concentrations as high amounts of it (100 U/mL) showed higher reduction. Samples incubated at 55°C showed no lane reduction compared to the respective control (Appendix B).

A comparison between the unheated and pre-heated samples shows that the disappearance of the α-Lactalbumin and β-lactoglobulin proteins in particular tended to increase with increasing enzyme concentration. However, the effect of enzyme concentration on α-Lactalbumin disappearance was not as marked for the pre-heated samples as it was for the unheated samples. The disappearance of the three caseins with increasing enzyme concentration was nowhere near as marked as it was for the two whey proteins. The results suggest that the pre-heat treatment had little effect on the disappearance of either the three casein or the two whey proteins and that enzyme concentration was the pre-dominant factor or determinant of how much protein would be cross-linked.

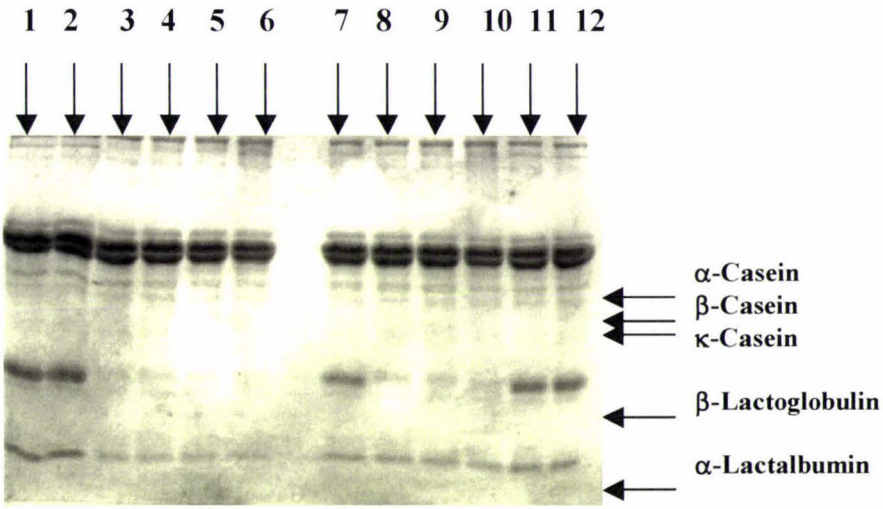

Figure 8: SDS-PAGE gel of Trim™ milk treated with transglutaminase (37°C for 2 h). Samples 1–6 were pre-unheated, and samples 7–12 were pre-heated at 90°C for 10 min. **1 and 2**: control unheated; **3**: 1 U/mL; **4**: 5 U/mL; **5**: 10 U/mL; **6**: 100 U/mL; **7**: 1 U/mL; **8**: 5 U/mL; **9**: 10 U/mL; **10**: 100 U/mL; **11 and 12:** control (heated).

4.2.1.2 Effect of heat treatment on Full Fat milk samples incubated with transglutaminase

An examination of The SDS PAGE pattern (Figure 9) for the Full Fat milk samples shows that the concentrations of the two whey proteins in particular were far lower in the pre-heated samples (lanes 7-12) than was the case for the unheated samples (lanes 1-6). In the case of both heat treatments whey protein disappearance appeared to be dependent on transglutaminase concentration with the highest reductions occurring at an enzyme concentration of 100 U/mL. The disappearance of the two whey proteins with increasing enzyme concentration tended to be more marked in the case of the unheated samples. The fact that substantial amounts of the two whey proteins had disappeared in the heated control samples (lanes 11 and 12) compared with the unheated controls (lanes 1 and 2) suggests that another mechanism was confounding the results for the pre-heated samples. Whey proteins are known to cross-link via a disulphide rearrangement reaction (Traoré and Meunier, 1991). These disulphide bonds are known to break and reform both inter- and intra-molecularly when proteins are heated above 68°C (Traoré and Meunier, 1991). It is possible that this latter reaction was responsible for the substantial cross-linking that was observed in the pre-heated samples compared to the controls. However, transglutaminase-mediated cross-linking of the whey proteins would appear to have also occurred in the pre-heated samples because the amount of the whey proteins that was removed in the pre-heated samples tended to increase with increasing enzyme concentration. However, the contribution of this latter reaction appeared to be less influential in the pre-heated samples than the disulphide rearrangement reactions.

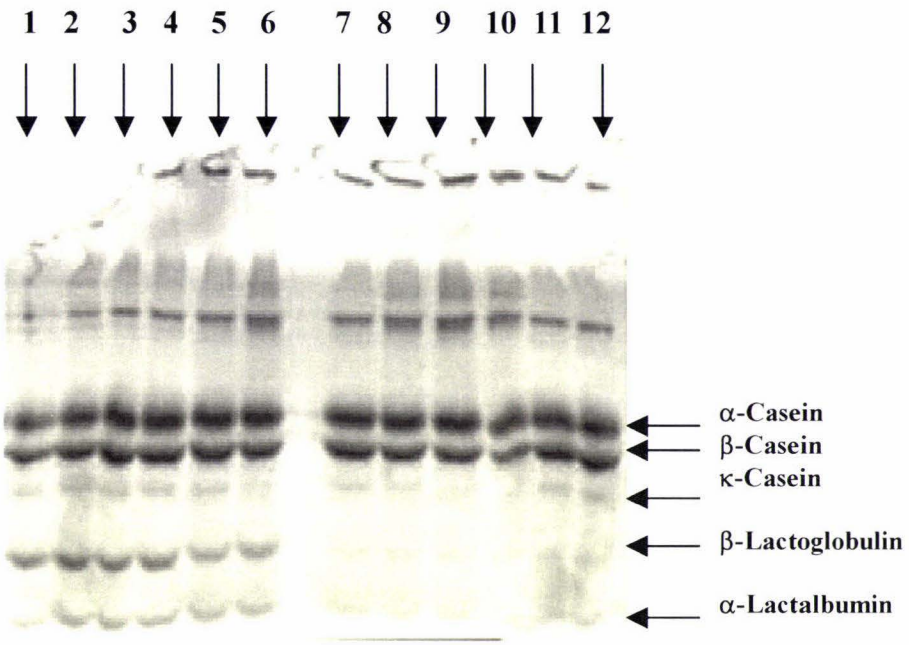

Figure 9: SDS-PAGE gel of Full Fat homogenized milk treated with transglutaminase (37°C for 2 h). Samples 1–6 were pre-unheated, and samples 7–12 were pre-heated at 90°C for 10 min. **1 and 2**: control unheated; **3**: 1 U/mL; **4**: 5 U/mL; **5**: 10 U/mL; **6**: 100 U/mL; **7**: 1 U/mL; **8**: 5 U/mL; **9**: 10 U/mL; **10**: 100 U/mL; **11 and 12:** control (heated).

4.3 Effect of Transglutaminase Incubation Temperature on Milk Samples

In this part of the research, two different incubation temperatures 37°C and 55°C were used. Time and the temperature of reaction are important considerations in the choice of conditions for any enzymatic reaction. A higher reaction temperature provided it does not exceed the enzyme's optimum temperature, leads to faster reaction rates and therefore less time is required to meet a specific degree of cross-linking. However, this depends mostly on the type of food to be processed and the desired physical properties of the final product (Ajinomoto, 1997). The SDS PAGE results from this experiment are shown in SDS-PAGE gel (Figure 9 (37°C) and Figure 10 (55°C). An examination of the unheated samples (columns 1-6) in both figures shows that whey protein reduction tended to occur at both incubation temperatures and that the amount of whey protein reduction tended to vary in an almost linear fashion with increasing transglutaminase concentration. Greater amounts of the two whey proteins were removed from the Full Fat milk systems

incubated at 37°C than at 55°C. This was evident for all enzyme concentrations. Greater amounts of β–casein were removed from the samples incubated at 37°C than was the case for the 55°C incubated samples. However, in the case of α-casein greater amounts of this protein were removed in samples incubated at 55°C than at 37°C, and the trend was particularly evident in the 10 and 100 U/mL.

As reported earlier greater amounts of the whey proteins and the three caseins were removed from the pre-heated samples (all enzyme concentrations including controls) than from the unheated samples. This trend was nowhere near as marked in the samples incubated at 55°C than it was for the samples that were incubated at 37°C. Greater amounts of the two whey proteins were removed from the preheated samples that were incubated at 55°C removed than was the case for the unheated samples. The same trend was evident for the three caseins as well. κ–Casein removal tended to be greatest when the samples were incubated at 37°C and the proportion of this protein that was removed tended to increase with increasing enzyme concentration. Removal of the other two caseins tended to be more pronounced at the lower incubation temperature than at the higher temperature. Furthermore, the decrease in the amount of these two proteins with increasing enzyme concentration tended to be more uniform for the samples incubated at 37°C compared to 55°C.

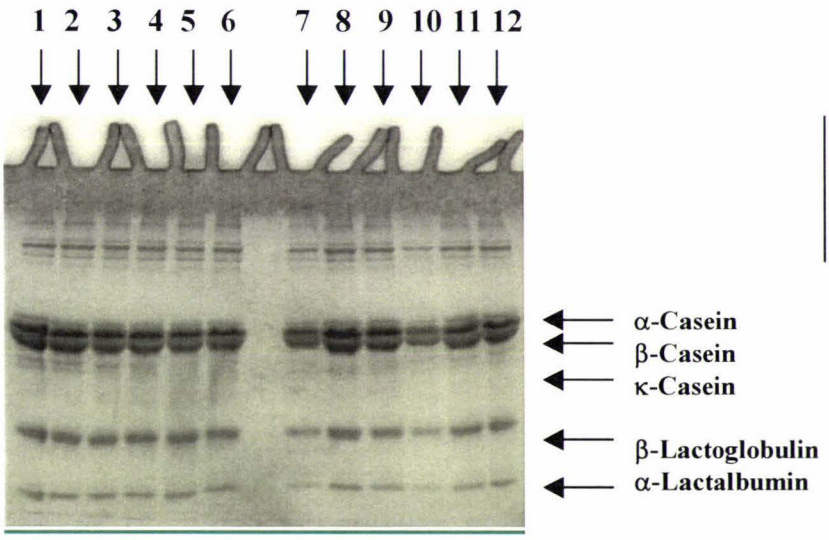

Figure 10: SDS-PAGE gel of Full Fat Milk homogenised and treated with transglutaminase (55°C for 2 h). Samples 1–6 were unheated, and samples 7–12 were heated at 90°C for 10 min. **1 and 2**: control unheated; **3**: 1 U/Ml; **4**: 5 U/Ml; **5**: 10 U/mL; **6**: 100 U/mL; **7**: 1 U/mL; **8**: 5 U/mL; **9**: 10 U/mL; **10**: 100 U/mL; **11 and 12:** control (heated).

With the experimental procedure used for this trial it was impossible to say whether the cross-linking of β-casein was due to self-association or transglutaminase cross-linking. The only way that the precise mechanism could be elucidated would be to chill the reacted milk to 4°C or less and to then do an SDS PAGE on the chilled milk to see whether the β-casein reduction that was evident in the incubated samples had persisted at the colder milk temperatures.

In summary it would appear that more transglutaminase cross-linking of milk proteins occurs when milk samples are incubated at 37°C than at 55°C.

4.4 Effect of Homogenisation On Full Fat Milk Samples Incubated with Transglutaminase

Figures 10 and 11 show the effects of varying the transglutaminase concentration (0 – 100 U/mL) on the behaviour of non-homogenized and homogenised Full Fat milk when incubated at 55°C. The non-homogenised samples (Figure 11) showed little cross-linking of the various casein and whey proteins. Furthermore, the cross-linking appeared to be independent of enzyme concentration (lanes 3-6 and 7-10). In the case of the homogenised samples (Figure 10) both casein and whey protein removal by cross-linking appeared to be more enzyme concentration dependent than the un-homogenised samples. During homogenisation it is known that some of the dairy proteins being examined in this study tend to lose their tertiary and quaternary structures when they form an inter-facial film between the fat droplets and the liquid medium. This protein film formation over the surface of the globules exposes lysine and glutamine residues, which are not normally available for reaction with non-homogenised milk. This exposure of the two amino acid residues on the surface of the fat globules means that transglutaminase mediated cross-linking is then possible. Consequently, one would expect to see more transglutaminase mediated cross-linking in full Fat milks that had been homogenized than in non-homogenised samples. This would suggest that homogenisation should be an integral step in any process to produce a transglutaminase modified whole milk product.

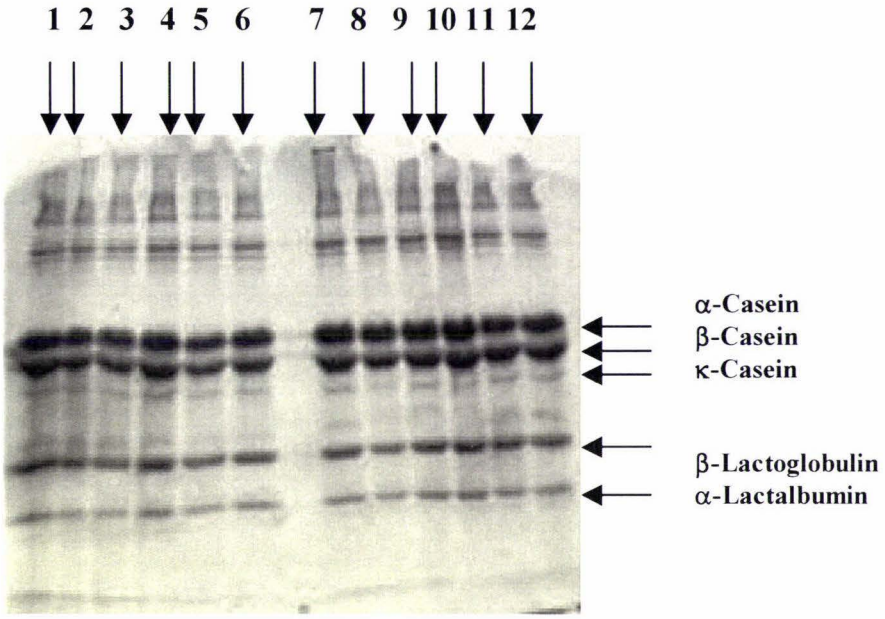

Figure 11: SDS-PAGE gel of Full Fat Milk non-homogenised and treated with transglutaminase (55°C for 2 h). Samples 1–6 were unheated, and samples 7–12 were heated at 90°C for 10 min. **1 and 2**: control unheated; **3**: 1 U/mL; **4**: 5 U/mL; **5**: 10 U/mL; **6**: 100 U/mL; **7**: 1 U/mL; **8**: 5 U/mL; **9**: 10 U/mL; **10**: 100 U/mL; **11 and 12:** control (heated).

4.5 Discussions

The pre-heat treatment trials on the Trim™ milk samples showed that pre-heating skim milk prior to the addition of transglutaminase had little effect on the cross-linking of either the casein or whey proteins when compared to the unheated samples. The results were similar to the results of Lorenzen and Qvist (2001) also those of Traoré and Meunier (1991). Lorenzen and Qvist (2001), in their study on the influence of transglutaminase treatment (bacterial transglutaminase similar to the enzyme used in this study) of skim milk on the formation of ε-(γ-glutamyl) lysine and the susceptibility of individual proteins towards cross-linking, showed that transglutaminase had little effect on the proteins in unheated milk, but cross-linking was considerable in the samples that were pre-heated. They also indicated that most of the cross-linking occurred in the first 30 min. For α-casein and β-casein major reductions in monomeric concentrations of both proteins were shown in both unheated and heated samples. However, Traoré and Meunier (1991) indicated that

heating caseins before enzyme treatment could result in greater molecular cross-linking. This was the case for casein when treated with Human Placental Factor FXIII, incubated with the milk at 50°C for 25 minutes. The products of the enzymatic reaction were analyzed by high-pressure liquid chromatography (HPLC). The elution patterns for the caseins, subjected to FXIII activated by Ca^{2+}, indicated that heating caseins prior to transglutaminase treatment did not increase polymerization by transglutaminase mediated cross-linking, although the heat treatment should have induced unfolding of the protein with a consequential unmasking of active glutamine and lysine residues thus leading to increased polymerization. These results suggest that preheating the milk prior to enzyme addition was not a compulsory treatment for Trim™ milk proteins that were to be covalently linked by transglutaminase. However, the results from Full Fat milk samples did show that greater removal of all proteins, and in particular the whey proteins, occurred when the two types of milk were preheated prior to the addition of transglutaminase. This removal was further enhanced in the Full Fat milk samples if the milk had been homogenized prior to the heat treatment. Proteins are known to denature when exposed to excessive heat or chemicals or high shear conditions in the presence of fat. The secondary and tertiary structures are destroyed and hydrophobic bonds and deeply buried groups are momentarily exposed. The same set of reactions can occur if proteins are homogenized in the presence of fat droplets. The presentation of these groups at the surfaces of adjacent proteins can lead to protein cross-linking via a number of reactions that include: hydrophobic bonds, salt bridges, hydrogen bonds and inter- and intra-molecular disulphide bridges. In the case of the disulphide bridges the pre-heat treatment can result in the cleavage of existing disulphide bonds or "activation" of buried sulfhydryl groups through unfolding of the protein. This results in the formation of new intermolecular disulphide bonds (bridging), which are required for the formation of a highly ordered gel structure in some protein systems (Boye *et al.*, 1995).

In this set of experiments significant amounts of protein aggregation or cross-linking was observed in the preheated samples compared to the controls. This was true for samples incubated at 37°C and 55°C. However, cross-linking via disulphide reformation or some other mechanism seemed to explain more of the cross-linking than could be explained by transglutaminase cross-linking. The heated controls for both samples showed significant removal of all proteins and in particular the whey

proteins when compared with the unheated controls, whilst increasing transglutaminase concentration had minimal effect on the cross-linking of both the caseins and the whey proteins. The lack of significant transglutaminase cross-linking of milk proteins could possibly be explained if the unfolding and subsequent exposure of the hydrophobic regions led to rapid aggregation of the proteins before the transglutaminase could access the exposed glutamine and lysine sites both within and between adjacent protein molecules (Traoré and Meunier, 1991). Traoré and Meunier (1992) in a later study showed that significant cross-linking could be achieved in preheated milk, and in particular of the whey proteins if the milk was heated with a reducing agent such as dithiothreitol (DDT) or glutathione or cysteine) before adding transglutaminase. The reducing agents prevented the rapid reformation of disulphide bridges and thus allowed the transglutaminase enough time to form cross-links with the exposed lysyl and glutamine residues. α-Lactalbumin cross-linking by transglutaminase was not affected by the presence of either of the above reducing agents. According to Traoré and Meunier (1992) disulphide bridges were not responsible for the reduced cross-linking observed for this protein in preheated milk when compared with unheated milk samples. They suggested that some other mechanism was limiting the amount of cross-linking that occurred between molecules of α-lactalbumin. According to them the unfolding of this protein on heating led to the exposure of hydrophobic regions on adjacent proteins and these aggregated to minimize the exposure of these hydrophobic regions to water molecules. If this was rapid the exposed glutamyl and lysine residues could become again hidden and thus be prevented from participating in any transglutaminase mediated cross-linking of the proteins.

Studies done by Faergemand and Qvist (1997) indicated that a partial unfolding of β-lactoglobulin is necessary in order to expose reactive glutamine residues; when using transglutaminase from *S. lydicus,* β-lactoglobulin to polymerise almost fully within a few hours. Aboumahmoud and Savello (1990), who used guinea pig liver transglutaminase when studying the polymerisation of β-lactalbumin, found polymerisation at much higher enzyme concentrations (almost 200 units/g protein) and longer reaction times than those used by Faergemand and Qvist (1997).

When looking at the effect of the enzyme concentration, the results indicated that there was a linear effect between the enzyme concentration and protein cross-linkage

as the bands tended to disappear gradually when the enzyme increased showing in most cases 100% reduction of β-lactoglobulin when incubated with 100 U/mL.

These results agree with studies carried out by Kuraishi *et al.*, (2001) in which reconstituted milks from whole and skim milk powder were treated with transglutaminase at 25°C for 2 h at different concentrations (0, 5, 10, 15, 20 and 25 units/g protein), giving higher cross-linkage at higher enzyme concentrations.

Meanwhile, there are two important factors that will determine the transglutaminase effect on milk. These are the enzyme incubation temperature and for full fat milk, the homogenisation process. As the results indicated, of the two incubation temperatures studied (37° and 55°C), both types of milk showed that the optimum incubation temperature for enzymic reaction and therefore protein cross-linkage was 37°C. At this temperature most proteins lanes on the PAGE gels tended to disappear noticeably compared to the respective controls. When investigating the interaction of transglutaminase with non-homogenised and homogenised Full Fat milk, we must first consider that this milk has a high amount of fat (4.0%) compared with Trim™ milk (0.42% fat). This fat-in-water emulsion has a membrane, with lipophilic and hydrophilic phases. As there is some evidence that the fat globule has a layered structure, it would be logical to suggest that the lipophilic membrane components would tend to lie close to the fat surface and that the outer layers would probably consist of hydrophilic components such as casein micelles (Visser *et al.*, 1991).

Our results clearly showed that homogenisation helped the protein cross-linkage (Figure 10), which when compared to non-homogenized sample (Figure 11) the protein reduction was considerably higher, and therefore more covalent bonds were formed. This could be because the homogenising effect occurs by forcing milk at high pressures through a very narrow orifice: the fat globules are deformed by turbulence, friction or inertia; they break suddenly and violently into pieces, while they are still in the gaps or after relaxation of the tension (Visser *et al.*, 1991).

When homogenisation occurs, a membrane of a different composition is formed on the freshly exposed fat surfaces. The average phospholipid content of the membrane decreases to as low as 10% of the original content, as presumably do the contents of many other components of the natural membrane (Visser *et al.*, 1991).

Mainly caseins and serum proteins replace these components quite rapidly. In other words, homogenisation can rupture the membrane by distortion of the fat globule.

However, such distortion can occur only when there is sufficient liquid fat present within the globule. When the membrane is ruptured, the fat globules are thought to escape and to act as an adhesive for binding fat globules together into clumps (Modler and Walstra, 1974).

The fat globule size distribution can affect the stability of the milk, and, the smaller the globule size, the more stable will be the emulsion. Thus, homogenisation is frequently used to prevent the rise of fat globules to form a cream layer. This stability and fat globule reduction could facilitate the protein-transglutaminase interaction when compared to non-homogenized samples.

4.6 Conclusions

The study showed that preheating Trim^{TM} milk and both homogenised and to a lesser extent non-homogenised Full Fat milk led to a substantial degree of aggregation of the whey proteins in particular and to a lesser extent the caseins in the milk samples. The fact that the preheated controls had also shown significant protein aggregation in comparison with the unheated controls suggested that some other mechanism, other than transglutaminase cross-linking, was responsible for this aggregation. The literature would suggest that the aggregation of the α-lactalbumin in the pre-heated milks were the result of inter-molecular disulphide bridges between adjacent molecules. The aggregation of β-lactoglobulin, on the other hand, could possibly have arisen from hydrophobic bonding. These other two cross-linking mechanisms inhibited the cross-linking by transglutaminase and thus little increase in aggregation was observed with increasing transglutaminase concentration in the preheated samples compared to the comparable unheated samples. The results would suggest that a preheat treatment was not necessarily favourable for protein–enzyme cross-linking for Trim^{TM} milk in particular.

The study showed that more of the whey proteins and β-casein were removed from Trim^{TM} milk that had been incubated at 37°C than at 55°C. This would suggest that future experiments should use an incubation temperature of 37°C to maximise the amount of protein cross-linking in milk products.

Homogenisation of Full Fat milk systems prior to any addition of transglutaminase appeared to have a beneficial effect on the cross-linking of the two whey proteins and three caseins when compared with the non-homogenised milk samples.

Homogenisation also appeared to enhance the aggregation of the whey proteins by both disulphide bridges and hydrophobic bonding according to the work of Traoré and Meunier (1992). Consequently, any future trials with milk systems that contained fat should be homogenised prior to the addition of transglutaminase to ensure that the maximum amount of transglutaminase-mediated cross-linking can take place.

Homogenisation increased cross-linking of milk proteins especially when this were pre-heated and incubated at high enzyme concentrations (100 U/mL) compared to non-heated and non-homogenized samples.

In conclusion, there was a positive response when milk proteins were incubated with transglutaminase (37°C for 2hr), as covalent cross-linkage was found especially at high enzyme concentrations (100 U/mL). The type of milk does not have an important impact in this protein-enzyme interaction, however, a pre-heat treatment and homogenisation seems essential for full fat milk.

CHAPTER V

5.0 EFFECT OF TRANSGLUTAMINASE ON MILK WITH PROTEIN CONCENTRATES

5.1 Effect of Transglutaminase on Milk with 0.5% of Sodium Caseinate

Caseinates are used widely throughout the world in food applications. Caseinates are valued particularly for their high solubility, bland flavour profiles, excellent emulsifying capacity, high fat and water binding, good whipping, heat stability, freeze–thaw stabilisation and foam stabilising properties.

The incubation of concentrated solutions of sodium caseinate or skim milk powder with Ca^{2+}-independent microbial transglutaminase leads to the formation of self-supporting gels of substantially different texture from those produced by conventional means (Nonaka *et al.*, 1992). Experiments on sodium caseinate gels with the addition of transglutaminase have shown that the casein in sodium caseinate cannot be seen in the SDS-PAGE gel electrophoresis lanes of the samples treated with the enzyme, the observation suggested that almost all the original casein components might be polymerised through to the isopeptide and might be too insoluble to form the network underlying the gel (Nonaka *et al.*, 1992).

In the previous chapter, experiments indicated that a heat treatment prior to the addition of transglutaminase could increased protein cross-linking. Therefore, all samples in the following experiments were subjected to a preheat treatment.

5.2 RESULTS

5.2.1 Effect of Incubation Temperature and Transglutaminase concentration on Trim™ Milk with 0.5% of Sodium Caseinate

In this set of experiments, 0.5% sodium caseinate (Chapter III, section 3.1.3) was added to Trim™ and Full Fat milk samples, which were then heated to 90°C for 10 min, cooled to either 37°C or 55°C and then treated with transglutaminase for 2h.

The effect of adding transglutaminase to Trim™ milk that was fortified with added sodium caseinate is shown Figure 12.

The 37°C samples with 1, 5 and 10 U/mL (lanes 2, 3 and 4) of added transglutaminase did not show any monomeric reduction compared to the control

sample (lane 1). However, when Trim™ milk was incubated with high enzyme concentrations (100 U/mL) (lane 5) almost 75% of the caseins were removed by transglutaminase-mediated cross-linking. Moreover, considerable reduction of whey proteins also occurred when compared to the control (lane 1).

The samples incubated at 55°C showed a very similar effect with increasing transglutaminase concentration as that demonstrated by the samples incubated at 37°C, with maximal protein removal occurring when the enzyme concentration was 100 U/mL. However, as can be seen from Figure 12 only 50 % of the caseins were removed at the higher incubation temperature and there was no clear trend with whey protein removal.

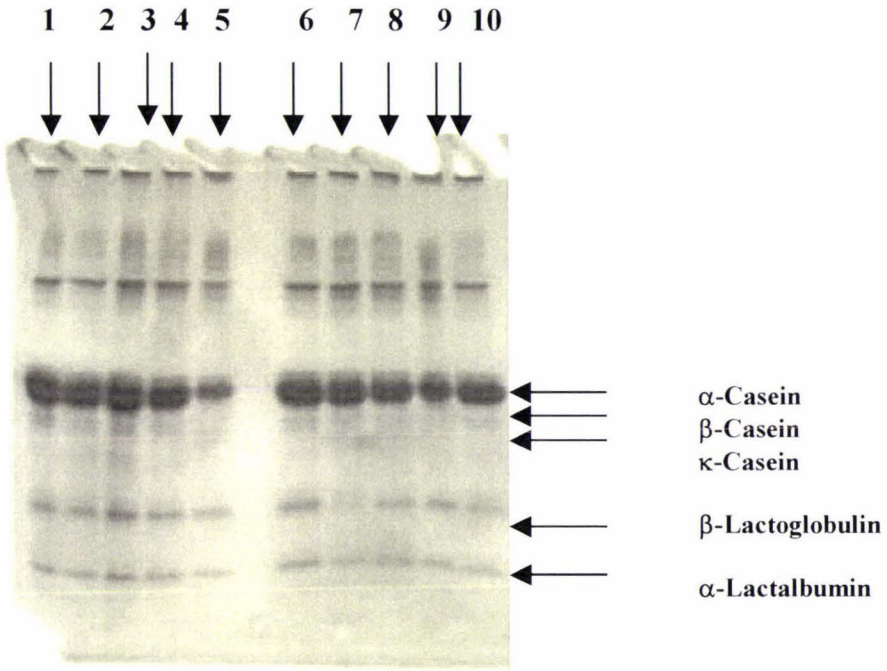

Figure 12: SDS-PAGE gel of Trim™ milk treated with transglutaminase and 0.5% sodium caseinate. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

The effect of adding 0.5% of sodium caseinate to the Trim™ milk can only be seen by comparing Figure 8 from Chapter IV and Figure 12 at 37°C. This comparison indicates that the degree of cross-linking of caseins in Trim™ milk was greater with sodium caseinate added than with no sodium caseinate addition when using 100 U/mL of transglutaminase.

The incubation temperature, as shown for skim milk alone, was a determinant factor in the results, with more caseins being cross-linked at 37°C than at 55°C.

5.2.2 Effect of Incubation Temperature and Transglutaminase concentration on Full Fat Milk (Homogenised and Non-homogenised) with 0.5% of Sodium Caseinate

Full Fat milk samples were firstly preheated (90°C for 10 min) and then homogenised at 60°C. Homogenised and non-homogenised samples were either incubated at 37°C or 55°C with varying concentrations of transglutaminase (0 – 100 U/mL). Figure 13 shows the SDS-PAGE gel, indicating that a major reduction was observed when non-homogenized milk was incubated at 55°C. Samples incubated at this temperature showed a gradual reduction as the enzyme concentration increased from 1 U/mL up to 100 U/mL, showing the maximum reduction (lane 9) compared to the control. This reduction was presented for caseins and whey proteins.

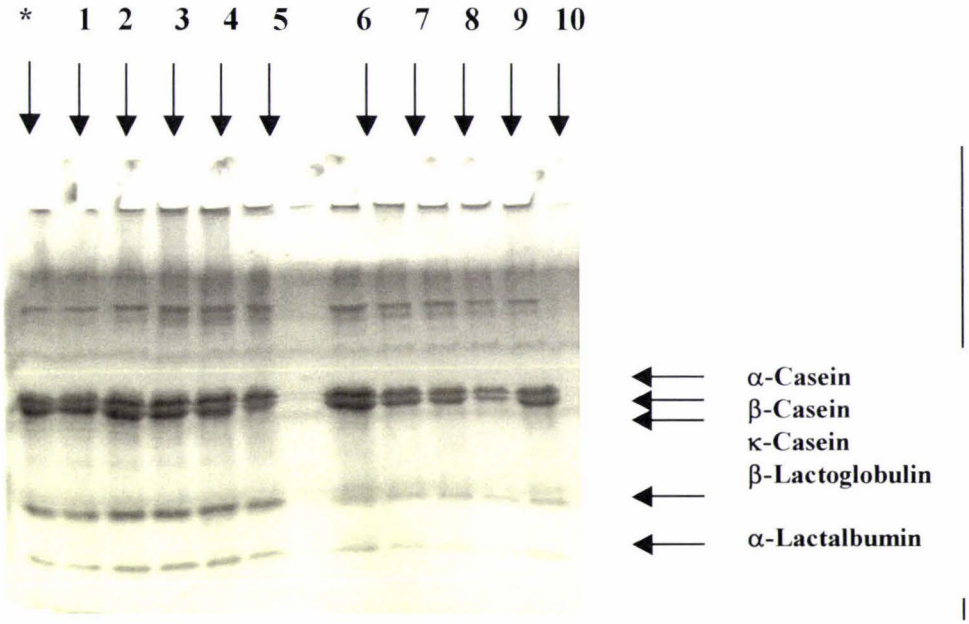

Figure 13: SDS-PAGE gel of Full Fat Milk non-homogenised treated with transglutaminase and 0.5% sodium caseinate. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) ***:** control replicate; **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

Clearly some aggregation or cross-linking of the non-homogenised sample incubated at 37°C did occur, but it appeared to be only at high enzyme concentrations.

The results suggest that casein and whey protein cross-linking can be maximised in a system of Full Fat milk with added sodium caseinate (0.5% w/w) by firstly pre-heating the milk to achieve significant denaturation of the heat labile proteins, then treating the milk with at least 100 U/mL of transglutaminase and incubating the milk at 37°C.

The results for the homogenized Full Fat milk samples are represented in Figure 14. The SDS PAGE results suggest that each of the protein bands tended to be reduced in the Full Fat milk samples incubated with transglutaminase at 37°C. Maximal reduction, i.e., 50% reduction of casein proteins, occurred at an enzyme concentration of 100 U/mL (lane 5). Similar results were obtained for β-lactoglobulin, but not much reduction occurred to the α-lactalbumin.

When milk samples were incubated at 55°C, the highest protein reduction (50%) occurred when only 10 U/mL of transglutaminase (lane 10) was added to the milk. The rest of the lanes had slightly smaller reductions in the various casein and whey proteins, but there was no definite trend of protein reduction with increasing enzyme concentration. In general, when comparing the effects of transglutaminase on homogenised and non-homogenised Full Fat milk, the results indicated that non-homogenized samples showed a significant reduction when incubated at 55°C, and this reduction was enzyme concentration dependent. Whilst 37°C proved to be the best enzyme conditions for protein cross-linkage in the homogenised samples. If the Trim™ milk and Full Fat milk results are compared it would seem that greater cross-linking occurred in the Trim™ than in the homogenised Full Fat milk samples which suggests that transglutaminase should be added to low fat systems rather than high fat milk systems.

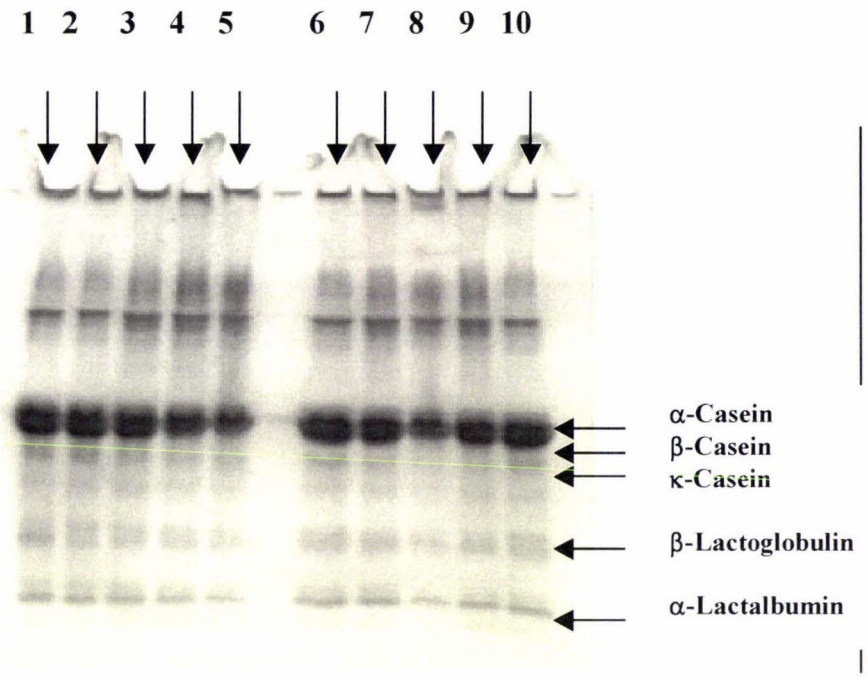

Figure 14: SDS-PAGE gel of Full Fat Milk homogenised treated with transglutaminase and 0.5% sodium caseinate. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

5.3 Effect of the addition of Transglutaminase on Milk Proteins and 0.625% Alacen 132

Alacen 132 (80% whey protein) is a soluble protein product, composed of α-lactalbumin and β-lactoglobulin that can form firm and elastic gels when heated. It is soluble over a wide pH range, and has thickening ability. It also has excellent nutritional qualities (protein efficiency ratio (PER) = 3.0).

This ingredient is particularly useful for yield extension and texture modification in various products such as processed meats, pasta, sausages, yogurts and desserts.

In this part of the research, the effect of the addition of whey proteins and transglutaminase to Trim^{TM} and Full Fat milk was evaluated. Most studies that have used transglutaminase have shown that the milk must be preheated before the transglutaminase is added; consequently this procedure was used for this set of experiments, with the Trim^{TM} and Full Fat milks being heated at 90°C for 10 min before being cooled to 37 or 55°C followed by the enzyme addition.

In general the results of these experiments indicated that the addition of 0.625% ALACEN 132 to milk samples incubated with transglutaminase had major effects on the whey proteins and especially on β-lactoglobulin in non-homogenised Full Fat milk samples incubated at 55°C.

5.3.1 Effect of Transglutaminase Incubation Temperature and Alacen 132 (0.625%) on Trim™ Milk

Figure 15 shows the SDS-PAGE gel of Trim™ milk with the addition of 0.625% Alacen 132 (Chapter IIIV, section 3.1.3) and transglutaminase at different concentrations and incubation temperatures (37°C and 55°C). The SDS PAGE gel shows that samples incubated at 37°C (lanes 1-5) lost significant quantities of the casein and whey proteins compared to samples incubated at 55°C (lanes 6-10). The protein-enzyme interaction increased as enzyme concentration increased and most protein reduction occurred when the enzyme concentration was 100 U/mL (lane 5) This lane shows a marked protein cross-linkage interaction of caseins, particularly β-casein, the monomeric form of which was almost eliminated.

High reductions were also shown by α- and κ-casein. The whey proteins were also significantly reduced in the samples that were incubated with transglutaminase at 37°C. The loss of the two whey protein monomers tended to be directly proportional to the concentration of transglutaminase in the sample. β-Lactoglobulin seemed to be a better substrate for transglutaminase, as it was reduced by nearly 100% in the treatment containing 100 U/mL of transglutaminase (lane 5, while α-lactalbumin showed little susceptibility to transglutaminase-mediated cross-linking.

A simple examination of an SDS-PAGE gel can neither quantify the cross-linking of β-lactoglobulin or the other proteins nor determine whether the interaction was caused by an increase in the intramolecular bonds between glutamine and lysine residues or by intermolecular bonding via rearranged -S–S- bonds or by a combination of both. For these reasons, a reduced SDS-PAGE gel was run, to determine whether the protein–protein interactions occurred by -S–S- bonds or by the transglutaminase cross-linking reaction (Figure 16). If a comparison is made of the 37°C incubation results (Figures 15 and 16, lanes 1-5) for normal and reduced SDS

PAGE of the Trim™ milk samples treated with transglutaminase of varying concentration it can be seen that the bands for the two whey proteins and κ-casein, which had almost disappeared in the normal SDS PAGE had reappeared in the reduced SDS PAGE Figure 16. The one exception was the samples treated with 100 U/mL of transglutaminase. In this sample both the 3 caseins and 2 whey protein bands were of comparable density to the normal SDS PAGE results. This suggests that any disappearance of the three caseins and two2 whey proteins in samples with transglutaminase concentrations below 100 U/mL were probably due to some form of –S-S- rearrangement both inter-and intra-molecularly. An examination of Figure 15 shows that the high molecular weight species that had not entered the gel lanes in the non-reduced SDS-PAGE gel had disappeared in the reduced gel indicating that the species were probably molecules that had been linked by covalent cysteine bonds. Whilst the disappearance of the five proteins from the samples treated with 100 U/mL of transglutaminase was probably due to transglutaminase-mediated cross-linking, as the thick band at the top of lane in each Figure had not changed in the reduced SDS PAGE Figure 16. It is also evident that additional protein bands appeared at a distance that was 20% from the top of the gel, and that the intensity of these bands increases with increasing transglutaminase concentration. At the same time as these bands were appearing the β-casein bands disappeared suggesting in part that the appearance of the high molecular weight species and disappearance of α- and β-casein were linked. κ-Casein disappeared completely at 100 U/mL.

The α-lactalbumin band also disappeared with increasing transglutaminase concentration, particularly at a transglutaminase concentration of 100 U/mL.

An examination of gel lanes 6-10 showed that at 55°C little or no cross-linking of any of the proteins occurred, though at 100 U/mL β-casein concentrations dropped slightly and a number of higher molecular weight species appeared at the 20% mark on the gel lane. Clearly 37°C is a more appropriate incubation temperature for transglutaminase mediated cross-linking of dairy proteins.

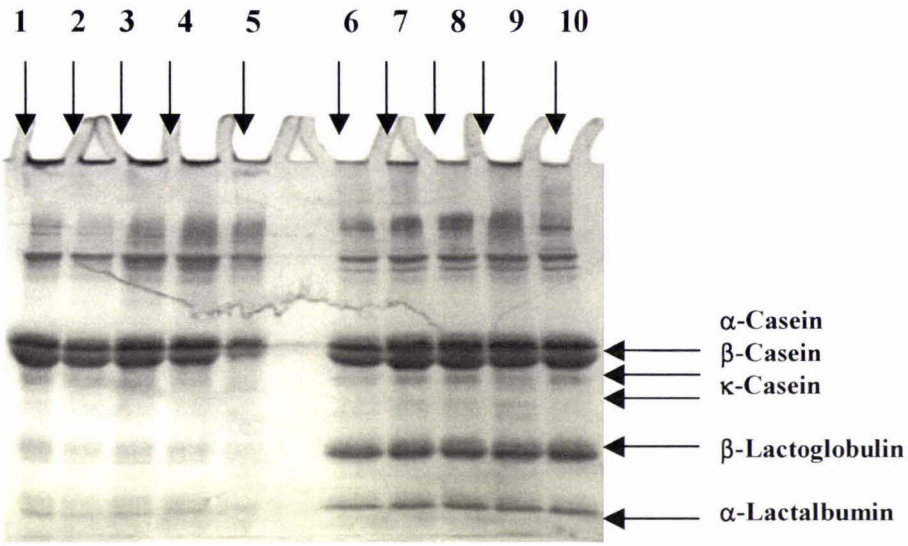

Figure 15: SDS-PAGE gel of Trim™ milk and 0.625% ALACEN and transglutaminase. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

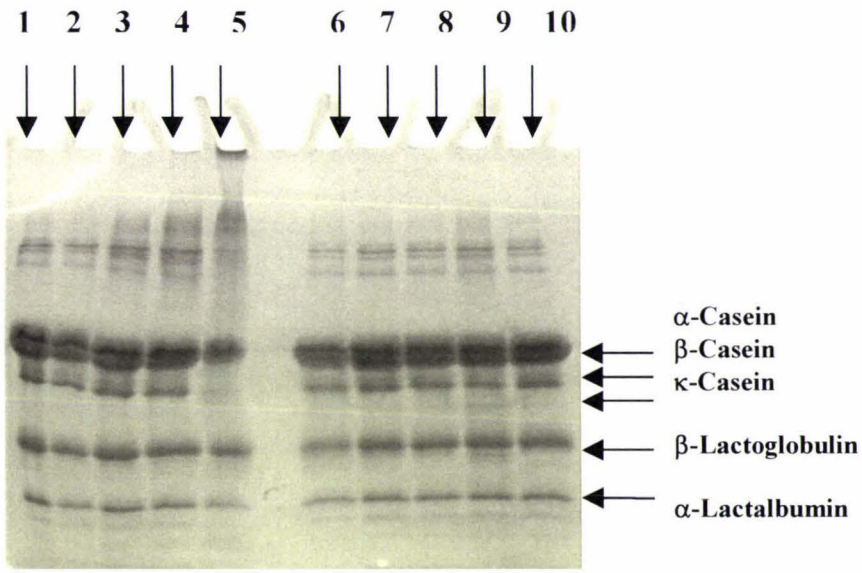

Figure 16: Reduced SDS-PAGE gel of Trim™ milk treated with transglutaminase and 0.625% Alacen. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

5.3.2 Effect of Transglutaminase Incubation Temperature and Alacen 132 (0.625%) on Full Fat Milk (non-homogenised and homogenised)

The effect of incubation temperature and transglutaminase concentration on Full Fat milk (non-homogenised and homogenised) was studied in milk samples pre-heated (90°C for 10 min). After the pre-heat treatment half the samples were cooled down to 60°C and homogenised, with posterior transglutaminase treatment either at 37°C or 55°C (0-100 U/mL).

An examination of Figure 17 shows that both casein and whey protein removal from the non-homogenised samples (lanes 1 – 5) tended to increase with increasing transglutaminase concentration, with most monomeric forms of each protein being removed at 100 U/mL (lane 5). Lane 5 shows that about 80% of β-casein and 70% of the κ-casein were removed during the transglutaminase reaction period. Comparable removals were also obtained for the two whey proteins as well. However, as no reduced SDS PAGE work was done for these gels it is impossible to say that transglutaminase-mediated cross-linkages were responsible for the removal of the whey proteins in particular. However, the fact that the caseins were removed from this system suggests transglutaminase was responsible for their removal as the caseins have very few cysteine residues that could participate in disulphide bridging with the added whey proteins. By comparison, samples incubated at 55°C showed no transglutaminase effect, as all lanes had very similar protein densities to the control (lane 10). The one exception was κ-casein, which appeared to be completely eliminated from lane 9 when the samples were treated with 100 U/mL of transglutaminase. Figure 18 shows the results for homogenised Full Fat milk samples with 0.625% of added Alacen and incubated either at 37°C or 55°C with transglutaminase (0-100 U/mL).

In these results it would appear that hardly any of the monomeric forms of either the caseins or the whey proteins were removed as a consequence of transglutaminase concentration or enzyme incubation period (lanes 2 – 8). However this gel shows that some protein was cross-linked by transglutaminase or some other mechanism as dense bands appeared on the top of each of the samples that were treated with transglutaminase (lanes 2 -9). The density of this layer at the top of each lane appeared to increase with increasing protein concentration and was highest for the samples treated with 100 U/mL.

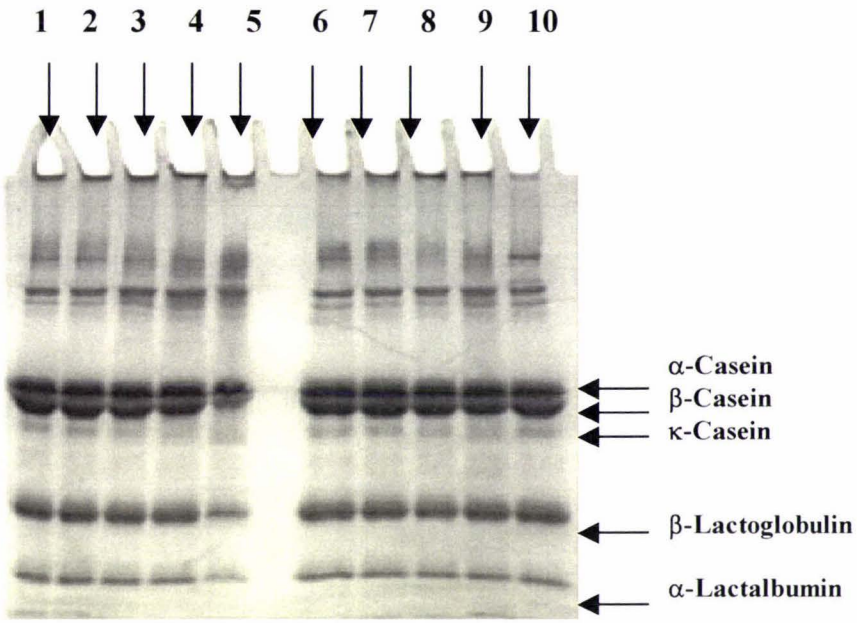

Figure 17: Full Fat non-homogenised milk SDS-PAGE gel treated with transglutaminase and 0.625% Alacen. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

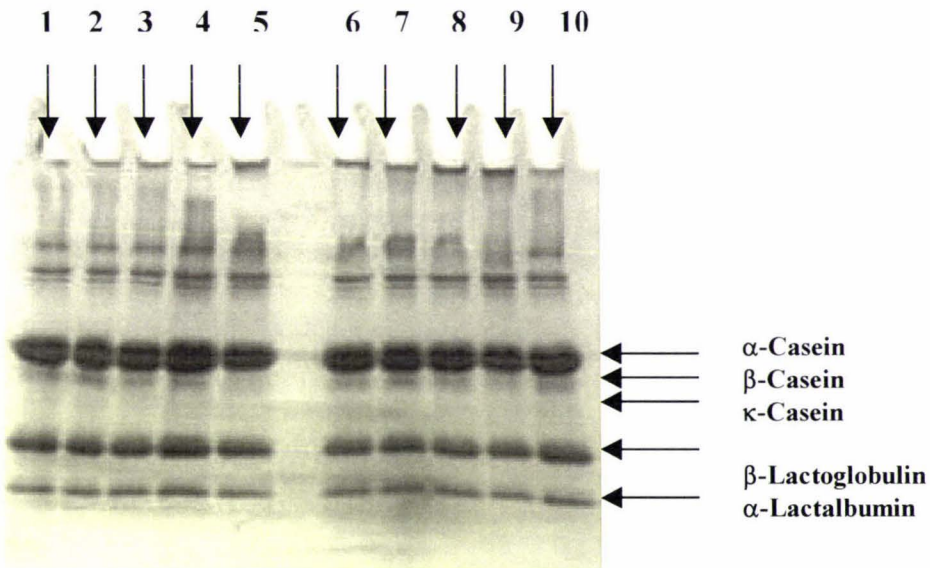

Figure 18: Full Fat homogenised milk SDS-PAGE gel treated with transglutaminase and 0.625% Alacen. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

Homogenisation of the Full Fat milk containing 0.625% of Alacen followed by incubation with transglutaminase appeared to have little effect on any of the proteins, even though there was a sizeable aggregated protein layer at the top of each lane that had been treated with transglutaminase.

5.4 Effect of the addition of Transglutaminase to Milk Proteins and 0.5% of Milk Protein Concentrates (MPC)

Milk protein concentrate (MPC) is a functional ingredient with standardised composition and is used as a source of proteins in the manufacture of a number of food products. Milk proteins are valuable ingredients, and are capable not only of replacing other proteins but also of enriching and improving the quality of the food products they are added to.

Caseins are found in the form of micelles in fresh milk (Euston and Hirst, 1999). Micelles are supra-molecular aggregates that consist of many caseins joined via calcium phosphate bridges. In contrast, whey proteins are more rigid, because their globular structure is governed by disulphide bonding, hydrogen bonding and hydrophobic interactions. During the processing of whole milk into protein products, the structure of the native micelle will depend on the type of process used to separate the various fractions.

Most MPCs are produced by membrane filtration. This type of milk concentrate is often called ultrafiltered MPC. One of its characteristics is that caseins and whey proteins remain in the same ratio as that in natural milk (Kameswaran and Smith, 1999).

MPCs are available in a range of protein concentrations from 42% to 85%. Depending on the protein concentration, MPCs may be used for a variety of purposes. For example MPC can be used as a substitute for skim milk powder of high protein content or as a reduced lactose milk product of high value. The extent of the denaturation of the whey protein in MPC depends upon the severity of the heat treatment employed during its production, which in turn can lead to quite marked differences in some of the functional properties of MPC.

Another characteristic of MPC is that, as the concentration of MPC is increased, the dissolution of some of the calcium phosphate associated with the casein micelles contributes to an increase in ionic calcium levels and, as the pH is reduced, calcium

from the micelles dissolves and contributes also to an increase in the ionic calcium levels.

Euston and Hirst (1999) compared the emulsifying properties of protein products containing aggregated and non-aggregated milk protein, and found that, because caseins are highly flexible, compared with whey protein, they are able to pack more efficiently at the surface when changing conformation. At low surface coverage, caseins adopt a flat extended conformation at the surface, whereas whey proteins cannot because their globular structure limits the extent of their deformation at the surface. Therefore, a similar situation could occur in MPC85, where the casein concentration is high and therefore a high packing fraction at the surface can be expected, because the casein molecules will adopt a conformation in which a larger proportion of the molecules extend into the bulk aqueous phase away from the surface.

Emulsions made with the large casein aggregates found in MPC85 were resistant to creaming compared with those made with sodium caseinate. This could have been because the protein aggregates found in MPC are large enough to cause significant flocculation, and therefore large protein aggregates have a relatively low osmotic pressure.

In this part of the research, the effect of the addition of MPC and transglutaminase to Trim^{TM} milk and Full Fat milk was studied. As a consequence of the addition of this concentrate, the samples would have had large casein micellar aggregates and a range of much smaller aggregates from the MPC.

5.4.1 Effect of Transglutaminase Incubation Temperature and Milk Protein Concentrate (0.5%) on Trim^{TM} Milk

An examination of Figure 19 (SDS PAGE), the results from the interaction of Trim^{TM} milk with MPC (0.5%) (Chapter III, section 3.1.3) in the presence of transglutaminase at different concentrations shows that the addition of transglutaminase to samples incubated at 37°C for 2 h caused the proteins to cross-link with each other, forming higher molecular weight chains that could not run down the gel. The disappearance of monomers is clearly seen in lanes 2 and 5, compared with the control sample (lane 1). According to the results in lane 5 (100 U/mL of transglutaminase) κ-Casein was the most cross-linked casein moiety.

At 55°C, the reduction in the quantities of the various monomeric forms of each protein was smaller than at 37°C and most lanes (6-9) had very similar lane patterns to the control sample (lane 10). This indicates once more that 37°C is the more appropriate incubation temperature for achieving significant transglutaminase mediated cross-linking of proteins. However, κ-casein reduction appeared to be proportional to the enzyme concentration and by the time the transglutaminase concentration had reached 100 U/mL there was no monomeric κ-casein left in the lane. With respect to the whey proteins, the SDS-PAGE gel results indicated that α-lactalbumin reduction was proportional to the enzyme concentration for both the 37°C and 55°C incubated samples. However, β-lactoglobulin, a supposedly good candidate for cross-linking by transglutaminase, showed little or no monomeric losses in this trial.

5.4.2 Effect of Transglutaminase Incubation Temperature and Milk Protein Concentrate (0.5%) on Full Fat Milk (non-homogenised and homogenised)

This trial evaluated the effect of incubation temperature and transglutaminase treatment on the reduction of various proteins in a Full Fat non-homogenized and homogenized milk proteins plus 0.5% added MPC system.

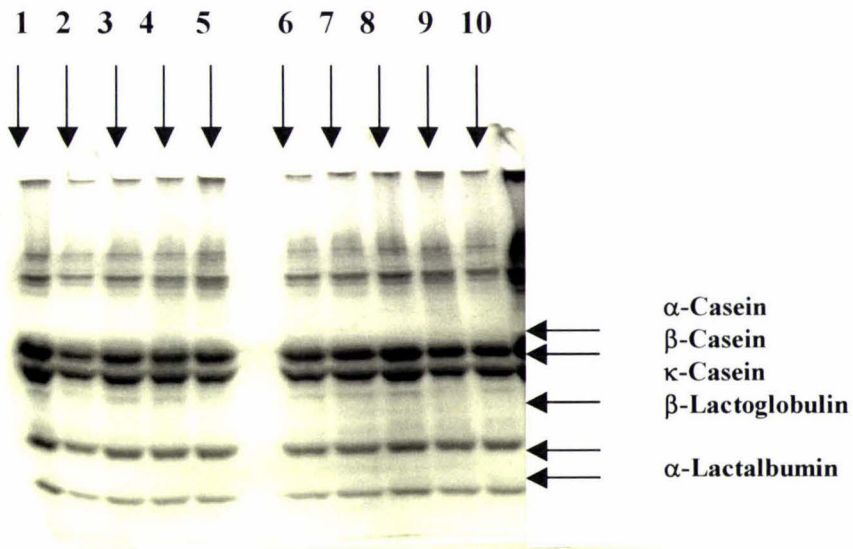

Figure 19: Trim™ milk SDS-PAGE gel treated with transglutaminase and 0.5% MPC. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

The non-homogenized samples showed to be predominantly affected by the enzyme incubation temperature and concentration as monomeric reduction were only shown at 37°C and the reduction trend was proportional to the enzyme concentration showing approximately 80% reduction when using 100 U/mL (lane 5) compared to the control sample (lane 1). This protein reduction was for all caseins and whey proteins. However, κ-casein and β-lactoglobulin disappear almost completely even at low concentrations (Appendix B).

The homogenized milk samples that were incubated at 37°C (lanes 1-5) show little loss of either the caseins or whey proteins with increasing transglutaminase concentration (lanes 2-5). Even the samples incubated with an enzyme concentration of 100 U/mL (lane 5) only showed a 10% reduction of the five proteins when compared with the control (lane 1). An examination of the protein bands in lane 5 (100 U/mL of transglutaminase) for the three caseins suggest that β- and κ-casein are better transglutaminase substrates than α-casein. Whey protein reduction followed a similar pattern to the three caseins with little being removed until the transglutaminase concentration had reached 100 U/mL, and even then only about 10% of the two whey proteins was removed.

Figure 20 highlights the changes that occurred in casein concentrations of this milk system. Given the experimental errors that are inherent with gel electrophoresis it would appear that incubation at 55°C with even 100 U/mL of transglutaminase had little or no significant impact on the concentration of caseins in the whole milk system, i.e., casein reduction was show to be insignificant until the enzyme concentration reached 100 U/mL. At this concentration the density of the protein band at the top of lane 5 was significantly greater than the control. It was significantly denser than the corresponding lane of the homogenized milk incubated at 37°C. The results for the homogenized milk plus milk protein concentrate were very similar to the results that were reported for homogenized milk fortified with Alacen.

5.5 Effect of the addition of Transglutaminase to Milk Proteins and 0.5% of Milk Protein Isolate

Milk Protein Isolate (TMP) is a spray-dried, flavor-reduced, soluble milk protein, manufactured by an exclusive process in which casein and denatured whey proteins are isolated together from fresh skim milk. The nutritive values and functional versatilities of the casein and whey protein are mutually enhanced to form a unique milk protein ingredient.

Some characteristics of TMP are that it has an excellent nutritional quality (PER = 2.8), and that it has very good emulsifying properties and foaming stability, high water binding capacity and solubility, and a low flavor profile. This ingredient can be used in coffee whiteners, whipped toppings, bakery products and nutritional products.

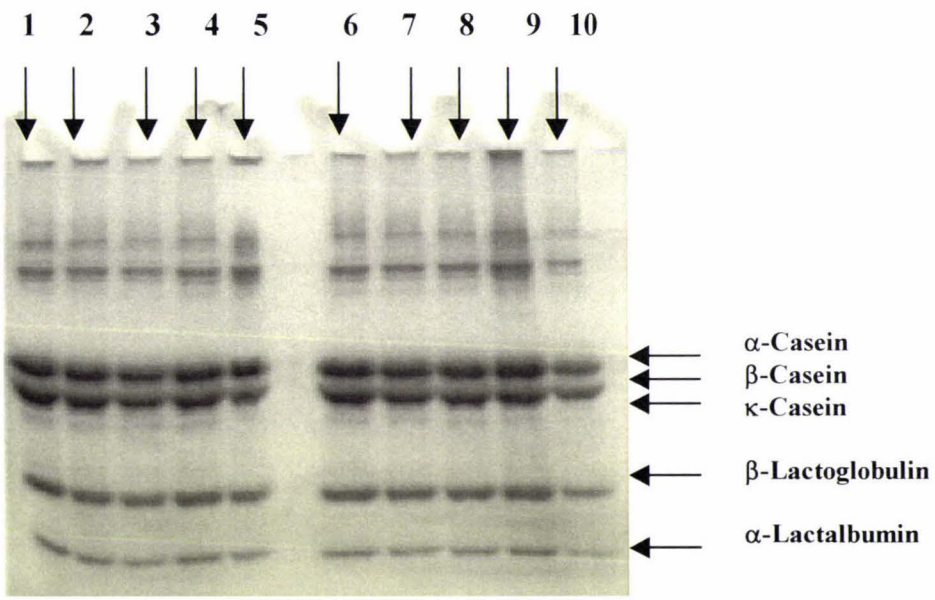

Figure 20: Full Fat homogenised milk SDS-PAGE gel treated with transglutaminase and 0.5% MPC. Samples 1–5 were incubated at 37°C for 2 h, and samples 6–10 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1:** control; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **10:** control.

5.5.1 Effect of Transglutaminase Incubation Temperature and Milk Protein Isolate (0.5%) on Trim™ Milk

The effect of incubation temperature and transglutaminase concentration on Trim™ milk with 0.5% of TMP (Chapter IIIV, section 3.1.3) was studied in preheated (90°C for 10 min) milk samples. After the pre-heat treatment half the samples were cooled down to 60°C and homogenised, with posterior transglutaminase treatment either at 37°C or 55°C (0-100 U/mL).

The results in Figure 21 show that when transglutaminase is incubated at 37°C at low enzyme concentrations (1 U/mL, lane 3), no protein cross-linkage was visible in the gel, as lane 3 looked almost identical to the control sample (lanes 1 and 2). However, samples incubated with 5, 10 and 100 U/mL did show the effects of transglutaminase-mediated cross-linking (lanes 4 -6) and the amount of protein removed was directly proportional with enzyme concentration. The highest amount of casein removal occurred in the milk that had been treated with 100 U/mL of transglutaminase. Whey proteins were also affected by enzyme however; β-lactoglobulin was shown to be a better transglutaminase substrate than α-lactalbumin (lane 6). Little or no cross-linking was evident in any of the transglutaminase samples that had been incubated at 55°C as evidenced by the lack of any substantial increase in the density of the protein layer at the top of each lane (7-12).

5.5.2 Effect of Transglutaminase Incubation Temperature and Milk Protein Isolate (0.5%) on Full Fat Milk (non-homogenised and homogenised)

The effect of transglutaminase and incubation temperature in Full Fat milk was investigated in non-homogenised and homogenised conditions.

Figure 22 represents the results for the non-homogenized milk samples. At 37°C transglutaminase appeared to cross-link the 5 main milk proteins extensively when only using 1 U/mL compared to the treatments with higher concentrations of transglutaminase and the controls. However, an examination of the protein layers at the top of each lane (1 -5) suggests that no more cross-linking occurred in the 1 U/mL sample than on the samples with higher enzyme concentrations. Given the quality of the gel it is hard to say whether increasing enzyme concentration had an effect on protein removal either for the 37°C or 55°C samples.

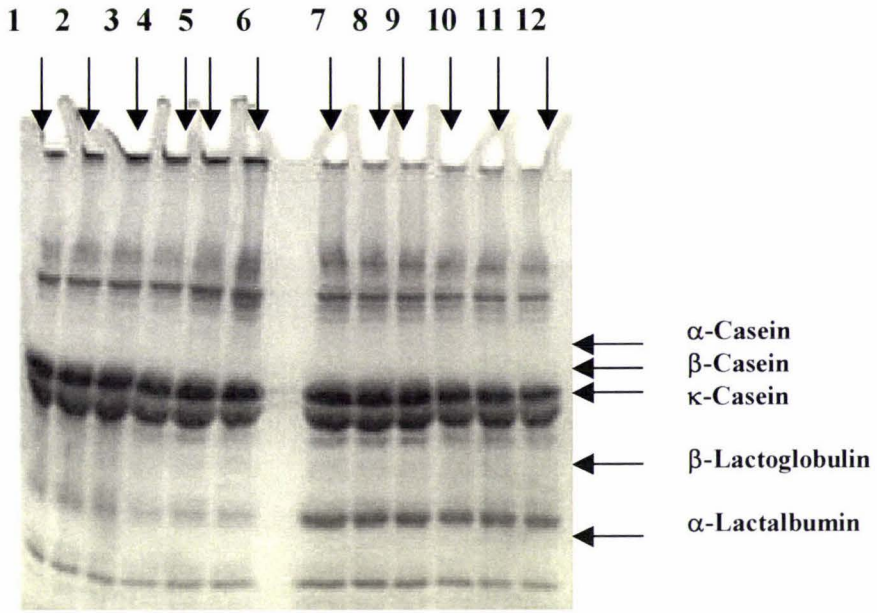

Figure 21: Trim™ milk SDS-PAGE gel treated with transglutaminase and TMP (0.5%). Samples 1–6 were incubated at 37°C for 2 h, and samples 7–12 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1-2** Control; **3**: 1 U/mL; **4**: 5 U/mL; **5**: 10 U/mL; **6**: 100 U/mL; **7**: 1 U/mL; **8**: 5 U/mL; **9**: 10 U/mL; **10**: 100 U/mL; **11-12:** control.

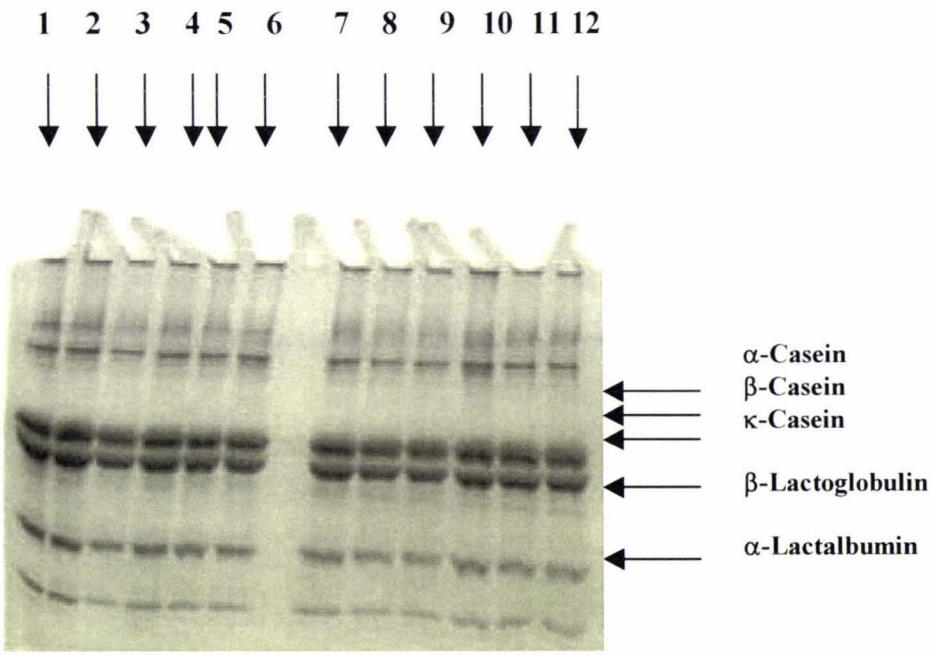

Figure 22: Full Fat non-homogenised milk SDS-PAGE gel treated with transglutaminase and TMP (0.5%). Samples 1–6 were incubated at 37°C for 2 h, and samples 7–12 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1-2** control; **3**: 1 U/mL; **4**: 5 U/mL; **5**: 10 U/mL; **6**: 100 U/mL; **7**: 1 U/mL; **8**: 5 U/mL; **9**: 10 U/mL; **10**: 100 U/mL; **11-12:** control.

Figure 23 shows the SDS PAGE gel for homogenized milk samples incubated at 37°C and 55°C with 0.5% TMP. The effect of transglutaminase on these proteins is clearly evident in lanes 3-6, where transglutaminase-protein interactions increase with increasing enzyme concentration when the samples are incubated at 37°C. β–Casein, κ–casein and β-lactoglobulin appeared to be the proteins most affected by the presence of transglutaminase in the homogenized milk system. The concentration of these proteins declined as enzyme concentration was increased. α-Casein did show some decline in concentration with increasing enzyme concentration, but the extent was nowhere near as high as it was for the other two caseins, α-Lactalbumin too declined with increasing enzyme concentration, but as with the alpha form of casein, the decline was not as extensive as it was for β-lactoglobulin.

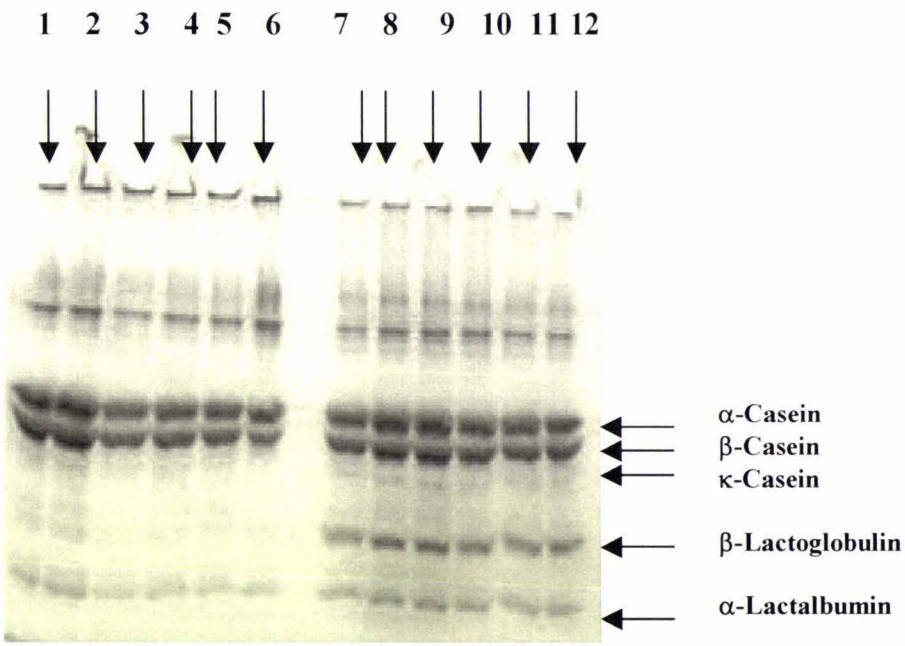

Figure 23: Full Fat homogenised milk SDS-PAGE gel treated with transglutaminase and TMP (0.5%). Samples 1–6 were incubated at 37°C for 2 h, and samples 7–12 were incubated at 55°C for 2 h. (All samples were pre-heated at 90°C for 10 min.) **1-2** control; **3**: 1 U/mL; **4**: 5 U/mL; **5**: 10 U/mL; **6**: 100 U/mL; **7**: 1 U/mL; **8**: 5 U/mL; **9**: 10 U/mL; **10**: 100 U/mL; **11-12:** control

The samples incubated at 55°C showed no major reduction in any of the 5 major milk proteins. The intensity of the protein bands in lane 7 (1 U/mL) were less than those in the other lanes 8 -12. However, as there was no increase in protein density at the top of this column compared to the others it can be assumed that the disparity in protein densities was probably a reflection of the how much protein entered the lane compared to the other lanes, and was not something brought about by transglutaminase cross-linking.

In previous milk systems the non-homogenized milk was shown to be more susceptible to transglutaminase cross-linking at the higher enzyme concentrations than the homogenized samples. However, in this trial the reverse was found, i.e., the homogenized samples were more susceptible to cross-linking than the non-homogenized samples, particularly when the enzyme and milk were incubated at 37°C rather than at 55°C. This would suggest that the added protein powder had a marked behavior on the sensitivity of the various proteins to transglutaminase-mediated cross-linking. It is possible that the process used to produce the various proteins had a significant impact on the ability of the proteins to unfold at the surface of fat globules and thus participate in protein-protein reactions that were mediated by transglutaminase. It is possible that the different proportions of the various proteins impacted on the functionality of the powder as well. Clearly, further experiments are needed to elucidate the exact mechanism that determines the functionality of the various powders that were used in this series of experiments could indicate that incubation temperature is a predominant factor on the enzyme efficiency, and more essential than homogenization.

5.6 Discussions

In the effect of transglutaminase treatment on Trim™ and Full Fat milk with different concentrations was investigated and it was found that transglutaminase cross-linked milk proteins predominantly at high concentrations (100 U/mL). To improve our understanding of the effect that transglutaminase has on milk proteins we proceeded to add different protein concentrates like sodium caseinate, Alacen, milk protein concentrates and milk protein isolate to verify the cross-linkage effect at

different incubation temperatures (37°C and 55°C for 2 h) and at different enzyme concentrations (0-100 U/mL).

The addition of sodium caseinate (0.5%) to milk samples treated with transglutaminase indicated that this protein concentrate could markedly improve protein cross-linking compared to just Trim™ milk samples (Figure 8 and 12) so long as the samples were incubated at 37°C. Sodium caseinate added at the same concentration as in the Trim™ had little effect on cross-linking of proteins in the non-homogenized samples. However, cross-linking of proteins, and in particular the casein monomers was significant in the homogenized samples, and the extent of the cross-linking tended to rise with increasing enzyme concentration in the samples incubated at 37°C. Cross-linking also occurred in the homogenized samples incubated at 55°C, but the degree of cross-linking was lower than in the samples incubated at the lower temperature, and also much lower than occurred in the non-homogenized samples incubated at 55°C.

The results for sodium caseinate appear to be at odds with results reported in Chapter IV and Nonaka et al., (1992) and Visser et al., (1991). According to these authors significant cross-linking of β-casein should have occurred in the homogenized samples because of its greater susceptibility to transglutaminase-mediated cross-linking, particularly in samples that had been homogenized. Further experiments should be conducted to see whether the difference between these results and those of other researchers is a 'real' result or simply an experimental error. Homogenization of the fat clearly had a significant impact on the casein molecules susceptibility to transglutaminase-mediated cross-linking. The increased surface area of the fat globules in the homogenized samples and their subsequent coating by casein molecules might have exposed lysine and glutamine residues that are normally buried inside the three casein molecules, which in turn could have led to enhanced cross-linking by the enzyme. Even though there were some problems with the quality of the data, sodium caseinate appeared to have some beneficial effects on the cross-linking of proteins compared to just Trim™ milk on its own with the enzyme.

The addition of Alacen 132 (0.625%), a source of whey proteins, appeared to increase the susceptibility source helped whey proteins to transglutaminase-mediated cross-linking in samples incubated at 37°C when compared with samples of Trim™ milk that contained no added whey proteins. This increase in cross-linking in the

samples with additional whey proteins could simply be because the concentration of these proteins had increased to reach some critical concentration that permitted significant cross-linking to occur (the more likely reason) or the added whey proteins had somehow facilitated their interaction in Trim^{TM} milk. However, after performing a reduced SDS PAGE gel on the Trim^{TM} milk samples with added Alacen 132 it was shown that the disappearance of the whey proteins could not be attributed to the transglutaminase, except possibly in enzyme concentrations of 100 U/mL, but was instead probably due to disulphide bonds instead of covalent bonds.

Full Fat milk samples did not show significant protein cross-linkage compared to Trim^{TM} milk. However it seems as though the non-homogenized samples incubated at 37°C with 100 U/mL did show some transglutaminase-mediated cross-linking.

The addition of milk protein concentrate (MPC 0.5%), micellar casein containing undenatured whey, gave similar results to those for Trim^{TM} milk fortified with Alacen 132. Better results were expected because MPC has a relatively high casein concentration, something that could normally be expected to encourage cross-linking of the caseins. A cross-linking performance approaching the samples with 0.5% added sodium caseinate was expected, but this did not eventuate. MPC is known to be composed of relatively large casein micelles, and the lack of expected enzyme cross-linking may have been due to the fact that these large micelles may not have been able to unfold adequately at the fat globule surfaces and so less than the expected amount of cross-linking occurred.

However, more cross-linking did occur in the samples with added MPC compared to the Trim^{TM} samples with no added MPC. This would suggest that a minimum amount of the two whey proteins must be present to permit extensive protein network formation by both inter-and intra-molecular bonding.

The results from this trial indicated that a transglutaminase concentration of 100 U/mL and incubation temperature of 37°C was needed to produce a significant reduction of the various milk proteins in both Trim^{TM} and non-homogenized Full Fat milk.

Protein cross-linking was expected to be enhanced by the addition of MPC to Trim^{TM} milk because casein and whey protein concentrations were boosted. This in turn should have meant that the minimum protein concentration for each of the major proteins should have been reached to permit good network development. The fact that the homogenized samples interacted more successfully with the added MPC

proteins suggests that the proteins in the MPC were not in a form to participate in transglutaminase-mediate cross-linking and that it was only after homogenization that the proteins were in a correct state to permit significant protein cross-links. It is possible that homogenization broke up the large casein micelles in MPC and thus permitted more lysine and glutamine residues to be exposed to the enzyme.

Lastly, the use of protein isolate (0.5%) on milk and the addition of transglutaminase at different concentrations indicated that some protein reduction at all enzyme concentration occurred in the Trim^{TM} and Full Fat samples incubated at 37°C The reductions were most significant when the enzyme concentration was 100 U/mL.

It was clear from the results that the SDS PAGE pattern for both Trim^{TM} and Full Fat milks depended on the type of protein powder that was added, the incubation temperature and of course the enzyme concentration. The composition of the powders and processing conditions used to manufacture the product in turn probably affected the functional properties of the various powders. Sodium caseinate at 0.5% appeared to be the most effective powder for enhancing transglutaminase-mediated cross-linking, particularly of the caseins.

Further work is obviously necessary to confirm the results, and also to cast some light on the different types of gels that are produced by the various proteins at various enzyme concentrations, incubation temperatures and processing conditions (homogenization). The aim of the suggested work would be to see why the various treatments had such a dramatic effect on the properties of the acid gels.

The study showed that β- and κ-caseins were the most susceptible proteins to transglutaminase presence and the best substrates at all enzyme levels and at 37°C. As mentioned before, caseins and whey proteins differ widely in structure and properties. One of the main differences is the relatively high content of proline residues in the caseins, which prevents a very close packed secondary structure. Whey proteins are less flexible than caseins and contain intramolecular S–S bonds that govern their more or less compact globular structure, especially that of α-lactalbumin.

For this reason, whey proteins were cross-linked by transglutaminase only at high enzyme concentrations (100 U/mL). However, it was very important to heat the milk prior to the enzyme addition so that the whey proteins could unfold and expose their amino acids for reaction.

In general, this research confirms that milk proteins, and especially β-and κ-casein, are good substrates for transglutaminase. The protein–enzyme reaction is markedly dependent on the incubation conditions, enzyme concentration and can be improved by the addition of a protein concentrate such as sodium caseinate.

This could lead to a large range of applications in the dairy industry, by creating new products via inoffensive enzymatic modification. Also, potential improvements in physical characteristics can be obtained by protein bonding, which is investigated in the next chapter.

5.7 Conclusions

For most trials the best conditions to maximize transglutaminase mediated cross-linking of specific milk proteins were an incubation temperature of 37°C together with a preheat treatment at 90°C for 10 min, to help the whey proteins in particular to unfold, expose their amino acids and interact with transglutaminase. However, incubation at 55°C was shown to be in some cases appropriate in non-homogenized Full Fat milk samples. The addition of protein concentrates to milk followed by transglutaminase treatment indicated that protein concentrates can help the protein cross-linking especially when using Full Fat milk, compared with pure milk samples. The results for the pure milk trials with transglutaminase were presented in Chapter IV.

Sodium caseinate was shown to be very good transglutaminase substrate as its addition to Trim^{TM} and Full Fat milk resulted in major reductions of the three casein proteins and the two whey proteins, particularly at a transglutaminase concentration of 100 U/mL. Alacen, a concentrate containing a high proportion of the two whey proteins, proved to be a less effective enhancer of casein cross-linking, but an effective enhancer of whey protein cross-linking, particularly at high transglutaminase concentrations. However, reduced SDS PAGE showed that much of the enhanced cross-linking was due to reformation of disulphide bridges. Transglutaminase mediated cross-linking of the five major milk proteins was not enhanced by the addition of MPC to any great extent. Even TMP was not a particularly effective enhancer of transglutaminase cross-linking, though the results for the 1 U/mL suggested that it was. Sodium caseinate would appear to be the most effective enhancer of transglutaminase-mediated cross-linking for both Trim^{TM} and Full Fat milk.

Studies should be carried out to find out why the various protein concentrates behaved so differently in the Trim™ and Full Fat milk systems. The studies should also investigate the properties of the various gels produced by the protein concentrates by using a range of techniques such as confocal microscopy, enzymatic hydrolysis and subsequent HPLC to investigate the nature of the cross-links and the structure of the gels.

When non-homogenized Full Fat milk, was used with sodium caseinate, nearly half of proteins were cross-linked by transglutaminase. However, homogenized samples showed less cross-linkage in most cases, with the exception of TMP.

The study showed that β- and κ-caseins were the most susceptible proteins to transglutaminase presence and the best substrates at all enzyme levels and at 37°C.

Whey proteins were cross-linked by transglutaminase only at high enzyme concentrations (100 U/mL). However, it was very important to heat the milk prior to the enzyme addition so that the whey proteins could unfold and expose their amino acids for reaction.

In general, this research confirms that milk proteins, and especially β-and κ-casein, are good substrates for transglutaminase. The protein–enzyme reaction is markedly dependent on the incubation conditions, enzyme concentration and can be improved by the addition of a protein concentrate such as sodium caseinate.

This could lead to a large range of applications in the dairy industry, by creating new products via inoffensive enzymatic modification. Also, potential improvements in physical characteristics can be obtained by protein bonding, which is investigated in the next chapter.

CHAPTER VI

6.0 APPLICATIONS OF TRANSGLUTAMINASE IN ACID MILK GELS

6.1 INTRODUCTION

Yogurt is a milk gel that is formed by acid fermentation of milk with lactic starter. The sensory and physical properties of this acid gel can be affected by heat treatment, reactions of calcium with milk proteins, homogenization conditions, type and quantity of stabilizer, acidifying rate, extracellular polysaccharides and, finally, protein content (Dannenberg and Kessler, 1988). The protein content of commercial yogurts is often boosted. There are many ways of achieving this, including evaporation, addition of non-fat dry milk solids or protein concentrates, and concentrating the milk by reverse osmosis (RO) or ultrafiltration (UF) (Biliaderis *et al.*, 1992). However, this practice results in a more expensive product and, possibly, a product with compromised sensory properties.

Commercially produced yogurts can suffer from syneresis, which is a spontaneous release of water from the gel accompanied by a reduction in volume. As no external forces are involved, syneresis can be brought about by changes in temperature and pH and by mechanical factors (such as vibration), and tends to get worse over time. When the problem is excessive, it often leads to dumping of product. Changes in temperature, vibration and physical impact, are three conditions that are often present in the distribution and storage of these products. A common means of minimizing this problem is to add a hydrocolloid, stabilizer and pectin. Although these additives often prevent syneresis, their addition often leads to a decrease in the desirable sensory characteristics of the product. Thus, an alternative method of preventing syneresis is needed. One possible and potential solution could be the use of transglutaminase, as this enzyme is known to form oligomeric filaments of casein in milk systems and these filaments should prevent the constriction of a normal acidic yogurt gel with time, temperature fluctuations and vibration.

Studies done by Lorenzen and Schlimme (1997) on the effect of enzymatic cross-linking on the texture of yogurt, indicated that enrichment of dry matter and/or of the protein content and addition of hydrocolloids are not sufficient for preventing syneresis of a yogurt gel. Thus, transglutaminase-catalyzed cross-linkage of protein

chains, could have an additional effect by stabilizing the three-dimensional network in yogurt.

The objective of this research was to investigate the effect of transglutaminase in acid milk gels on syneresis and textural properties, as well as the enzymatic effect enzyme when left TGase active in acid milk gels during storage.

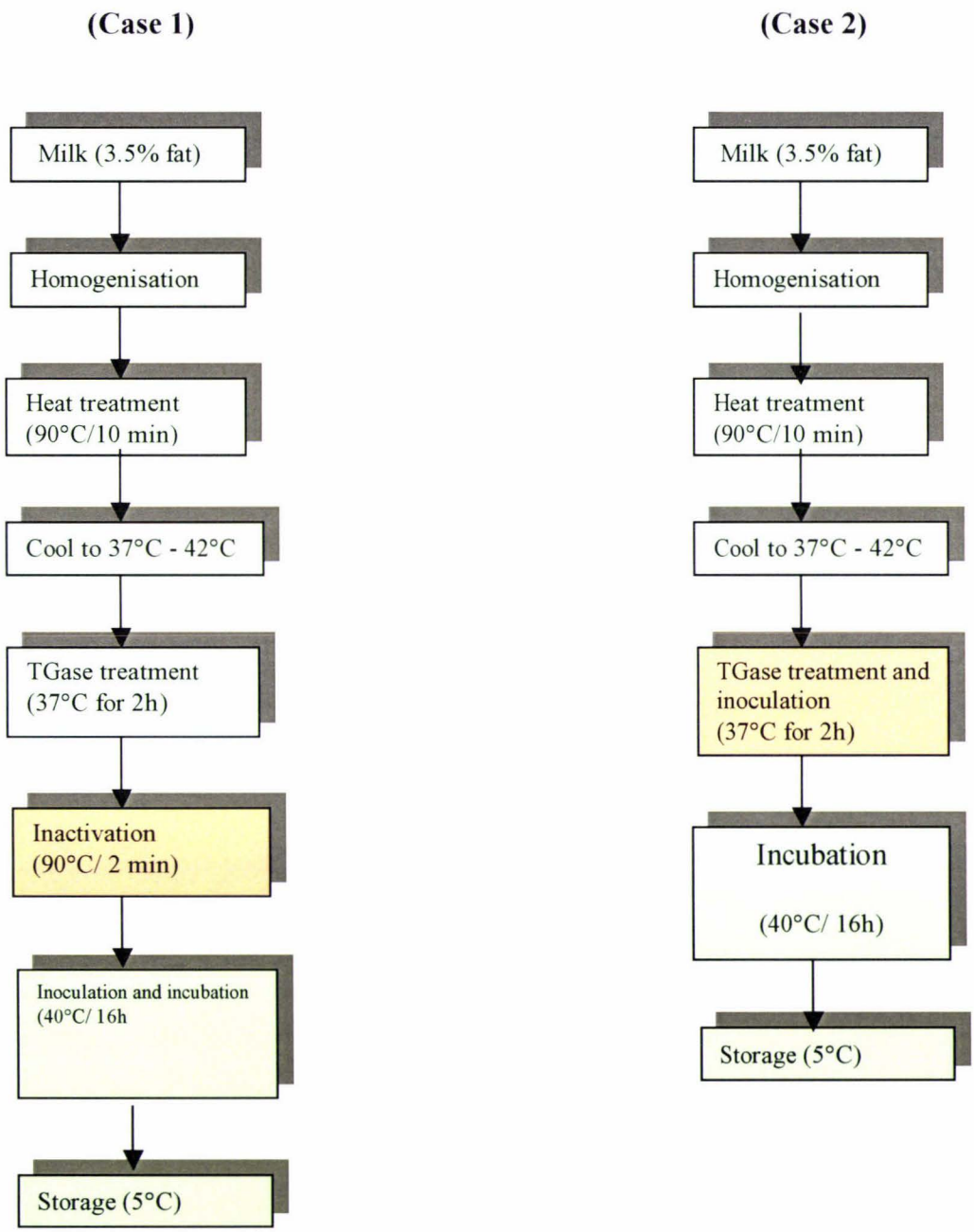

Figure 24: Enzymatic treatment of acid milk gels

6.2 RESULTS

6.2.1 Acid Milk Gels Treated with Transglutaminase

The objective of this trial was to investigate the effect transglutaminase cross-linking of milk proteins would have on the gel strength and reduction of syneresis of yogurt systems. Milk samples were pre-heated at 90°C for 10 min, to ensure that a substantial proportion of the serum proteins were largely denatured which in turn would ensure that a higher proportion of protein could participate gel formation and interaction with the enzyme could be significant. After cooling to 37°C, transglutaminase was added at different concentrations (0, 10, 50, 100 and 200 U/mL) and incubated for 2 h in a water bath. The various samples were then divided in two and in one half (case 1) the transglutaminase was inactivated by heating the milk to 90°C for 2 minutes in a microwave, cooling it and then adding the starter. A normal yogurt procedure (incubation for 16hr at 4°C) was then performed.. In the case of the other half of each solution (case 2), the enzyme was added together with the starter, incubated for 2 h at 37°C and this was followed by a normal yogurt procedure (Figure 24) with no inactivation (Chapter III, section 3.2.7).

Figure 25 shows the free whey in yogurt treated with transglutaminase at different concentrations for case 1 and case 2. The first thing to notice about the results is that the samples that were given the enzyme inactivation step (case 1) all showed higher whey losses than the samples that did not receive the inactivation step (case 2). The differences were particularly marked for those samples that had been treated with 100 U/mL. Even the control samples showed this difference suggesting that repeated heat treatment of milk systems can lead to increases in the amount of free whey in yogurt. Minimum whey loss (0.5g) in the inactivated system (case 1) occurred at enzyme addition rates of 50 U/mL, whereas minimum losses (0.06 g) for the non-inactivated enzyme system (case 2) occurred at an enzyme concentration of 100 U/mL. Both treatments showed a concave relationship between enzyme concentration and whey losses with weigh losses increasing both above and below the indicated optimum enzyme concentration. This would suggest that an optimum number of cross-links need to be formed in the yogurt to stabilise the system and if insufficient cross-links are formed (enzyme concentration below optimum) then whey will not be trapped in the gel network adequately to prevent loss. However, if greater numbers of cross-links than the optimum are formed then this could lead to excessive contraction of the

gel network, once again leading to increased whey losses (see Appendix C4 for more details.

As a consequence of the differences in whey loss between the controls, it is difficult to say that the improvements in whey loss that were observed between the two treatments were due solely to the fact that the transglutaminase continued to act in the non-inactivated controls during the overnight storage, and so formed more cross-links at each enzyme concentration than the inactivated samples, or whether the proteins in the inactivated samples had undergone further denaturation in the second heating stage and this had affected their whey binding properties compared to the non-inactivated samples. The differences between the treatments can probably be ascribed to both of the above mechanisms and further work is required to elucidate the precise mechanism that led to the observed results.

Physical characteristics such as gel strength were also studied in this research (Chapter III, section 3.3.2). The results (Figure 26) indicated that gel strength of acid milk gels was increased by the addition of transglutaminase and that the gel strength increased asymptotically with increasing enzyme until some optimum was reached at which stage gel strength started to decline. The first noticeable increase in gel strength occurred in the still active system (case 2) when 50 U/mL enzyme, yogurts were some 33.3% higher than the controls. Whilst the gel strength of the non-inactivated yogurt at an enzyme addition rate of 100 U/mL was almost double that of the controls (Appendix C7). It would appear from these results that a more substantial network of cross-linked protein is necessary for gel strength improvement than was needed for whey loss prevention.

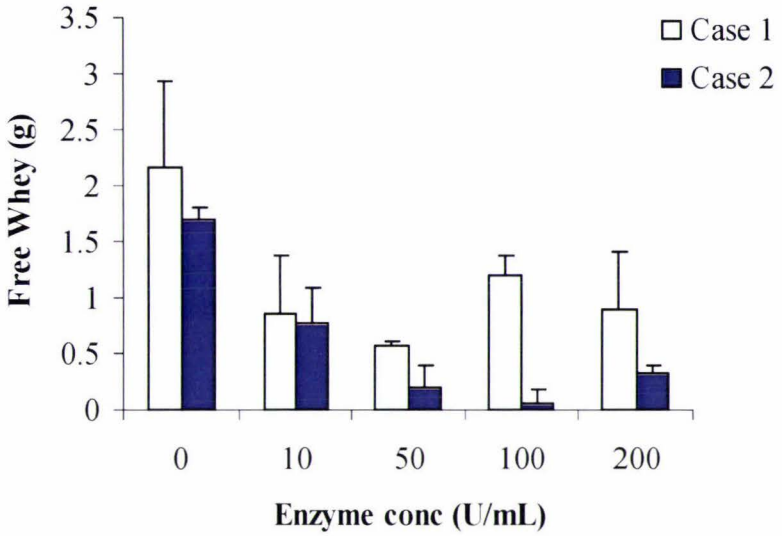

Figure 25: Free whey in acid milk gels treated with transglutaminase.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

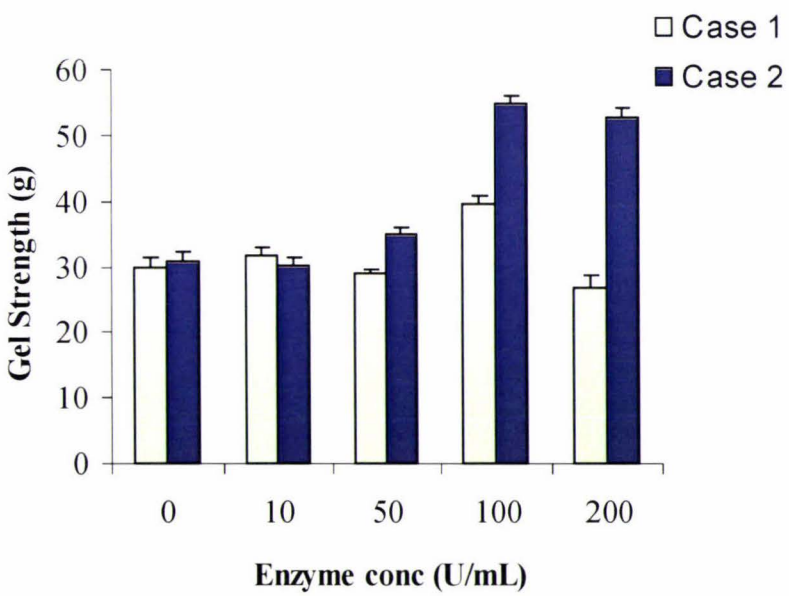

Figure 26: Gel strength in acid milk gels treated with transglutaminase.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

6.2.2 Acid Milk Gels Treated with Transglutaminase and Sodium Caseinate

In this experiment, sodium caseinate (0.5%) was added to the yogurt mix to determine whether it would prevent syneresis and improve the physical characteristics of the transglutaminase-treated samples incubated at 37°C. A similar experimental design to that used in the previous section was used here. (Chapter III. section 3.2.7).

Figure 27 shows the amount of free whey that was released from the inactivated (case 1) and non-inactivated (case 2) acid milk gels with added sodium caseinate (0.5%). As with the pure skim milk samples differences in whey holding power were observed between the 2 controls with whey losses from the non-inactivated samples being almost 57% lower than the inactivated controls. The added casein appeared to have a dramatic improvement on the whey loss with enzyme concentrations as low as 10 U/ml, leading to an almost 100% prevention of whey loss from both treatments. The inactivated yogurt followed a flattened concave relationship with enzyme concentration with losses being higher at 10 and 200 U/ml, than at the intermediate enzyme concentrations (see Appendix C5). The non-inactivated samples showed complete whey prevention at all enzyme concentrations used in the study. The results suggest that the added sodium caseinate led to a substantial increase in the network formation of the gel, even at low enzyme concentrations and that this improved network formation prevented the loss of whey when comparing these results with the yogurts that had no added casein. These results confirm that sodium caseinate is an excellent protein booster for acid milk gels if transglutaminase is used.

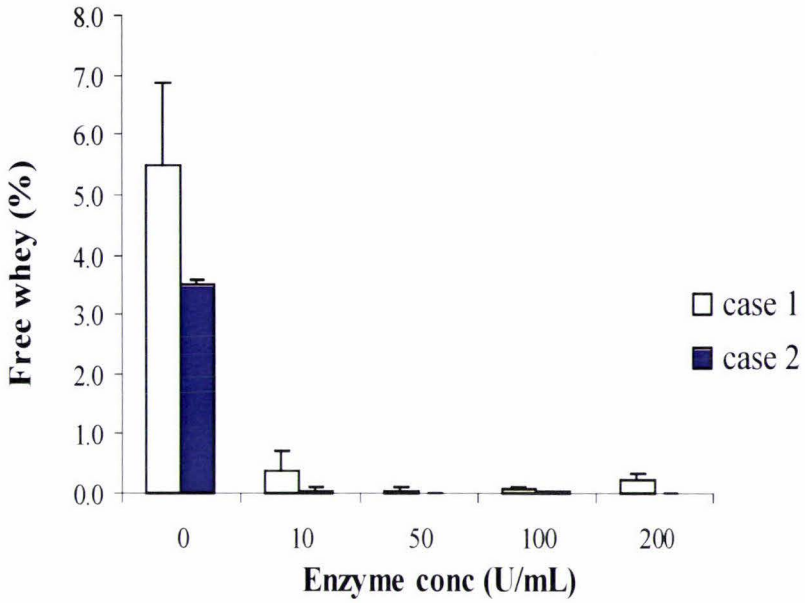

Figure 27: Free whey in acid milk gels treated with transglutaminase and 0.5% sodium caseinate.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

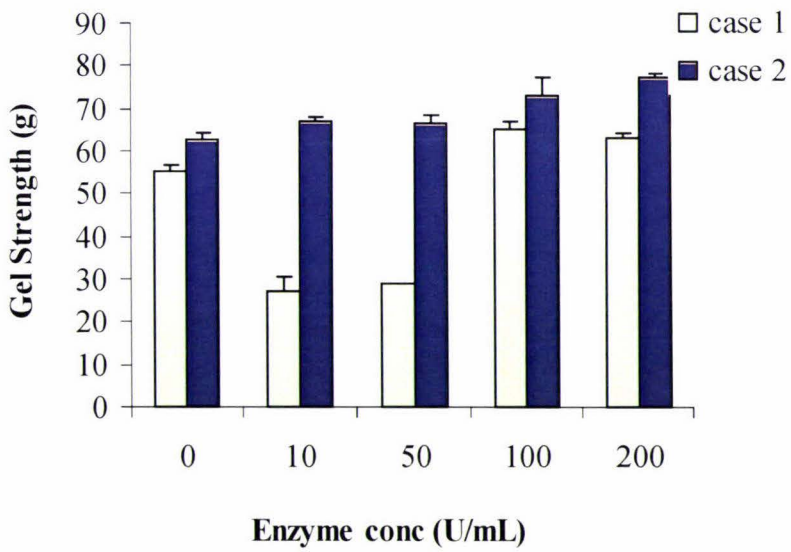

Figure 28: Gel strength in acid milk gels treated with transglutaminase and 0.5% sodium caseinate.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

Figure 28 and Appendix C8 show the gel strength results for the 2 treatments. The results for the two treatments were dramatically different. The gel strength for the inactivated samples showed a concave relationship with increasing transglutaminase concentration. Gel strength was a minimum for enzyme concentrations between 10 - 50 U/mL before rising to a maximum at an enzyme concentration 100 U/m. The non-inactivated samples showed a very slight increase in gel strength with increasing enzyme concentration. From a statistical standpoint the increase in gel strength with increasing enzyme concentration was insignificant. The results for the non-inactivated samples were quite different to those reported for non-fortified skim milk yogurts (previous section), and suggest that the skim milk systems were deficient in the caseins, and as a consequence adequate network formation was not possible unless high enzyme concentrations were used (100 U/ml.). Whereas in the fortified yogurt system there was sufficient raw material for adequate network formation to form to have a dramatic effect on the texture, even when only low enzyme concentrations were used (10 U/ml.).

6.2.3 Acid Milk Gels Treated with Transglutaminase and Milk Protein Isolate

In this experiment the objective was to study the release of free whey and the improvement in gel strength of yogurt as a consequence of the addition of 0.5% of milk protein isolate (TMP). The same experimental procedure that was used for the previous two experiments was used in this experiment (Chapter III, section 3.2.7).

Figure 29 shows the free whey (g) released from the two treatments. Unlike the previous experiments little difference was observed between the two controls. It would appear therefore that something in the added TMP was protecting the proteins from releasing excess whey after the second heat treatment when compared with the non-inactivated controls. Alternately, there was something in the TMP that inhibited extra network formation in the non-inactivated samples compared to the controls. Further work would appear to be required, such as electro-focusing to find out why TMP produced different effects in the yogurt system compared to skim milk or skim milk with sodium caseinate.

Both treatments showed a concave relation between free whey and increasing transglutaminase concentration, with both systems showing a minimum whey loss at a

transglutaminase concentration of 100 U/mL (Appendix C6). The amount of free whey released from the inactivated controls increased dramatically if the enzyme concentration was raised to 200 U/mL, but no such increase was observed for the non-inactivated samples.

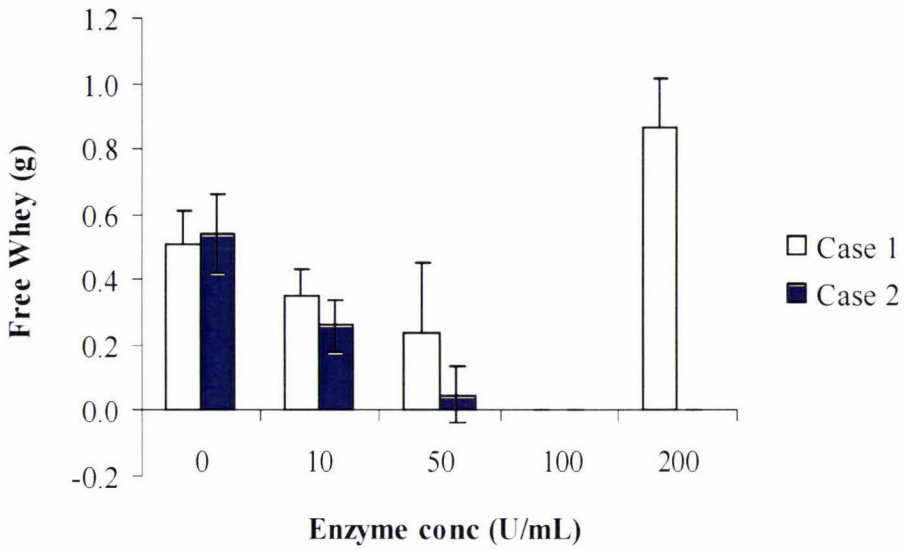

Figure 29: Free whey in acid milk gels treated with transglutaminase and 0.5% Milk protein isolate.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

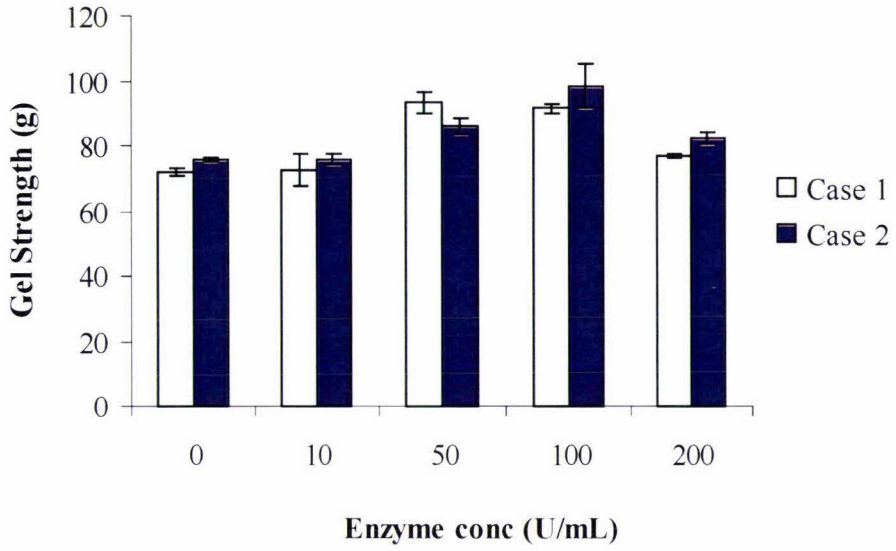

Figure 30: Gel strength in acid milk gels treated with transglutaminase and 0.5%Milk protein isolate.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

Figure 30 shows the changes that occurred in gel strength with increasing concentration of transglutaminase. in acid milk gels with TMP (0.5%). Maximum gel strength was found at concentrations of 50 and 100 U/mL (93.36 and 91.4 g) respectively for case 1 and (86.17 and 98.50 g) respectively for case 2 (Appendix C9).

6.2.4 Shelf Life Behavior of Acid Milk Gels Treated with Transglutaminase and Sodium Caseinate

In this set of experiments 1% of sodium caseinate was added to the skim milk and the milk was then treated with various concentrations of transglutaminase (0, 50 and 100 U/mL) and then incubated at 37°C and 55°C for 2 hours. At the end of this period the samples were divided in 2 and half was given a further heat treatment (case 1) of 2 minutes in the microwave to inactivate the transglutaminase before being cooled and having the yogurt culture added. The remaining milk was cooled and then the yogurt culture was added. A series of control samples were also included in the experiment. These had no added caseinate, but half were given the inactivation treatment as for case 1 and remainder were treated as for case 2. The transglutaminase in these samples was then inactivated before starter was added in exactly the same way as was described in section 5.2.1. The remaining half of the sodium caseinate enhanced

milk was treated with the same concentration of transglutaminase in conjunction with starter culture. The yogurt samples were then stored in a chiller for 2 weeks at 5°C. The loss of whey and the gel strength of the samples were followed over this two week storage period to see what effect storage had on the above physical properties of yogurt.

Figure 31 shows the results of samples incubated at 37°C, indicating clearly that the use of transglutaminase at any concentration helped to reduce free whey compared to samples with no added enzyme (Appendix D1). Significant whey loss occurred in samples with no added sodium caseinate. The losses were particularly marked after 2 weeks storage for both treatments. Whey loss tended to decrease for both treatments with increasing transglutaminase concentration, with least whey being lost in the samples treated with 100 U/mL of transglutaminase. Storage time had little impact on the loss of whey from the yogurt samples fortified with added sodium caseinate. There were no significant differences between the samples subjected to inactivation or non-inactivation over the two week storage. Clearly, the added sodium caseinate had a dramatic effect on the whey loss properties of the yogurt systems. This effect seemed to be the dominant determinant of whey loss and adding transglutaminase to a system with 1% added sodium caseinate appeared to have no significant impact on whey loss. This would suggest that so long as the casein levels are high enough in yogurt systems there should be no problems with whey loss. However, production costs may not permit such a high level of solids addition

Appendix D2 shows the results for the sample incubated at 55°C. The results indicated that free whey release could be reduced by incrementing the enzyme concentration and that minimal whey loss occurred for both treatments, i.e., 100% free whey reduction for case 1 and case 2 at the first week when the samples were treated with 100 U/mL, while in week 2, only case 2 showed a complete reduction, leaving case 1 with half of reduction compared to the control sample which showed 4g of free whey.

Table VI shows the results of gel strength for samples incubated with transglutaminase at different concentrations, temperatures and storage time. The results showed that gel strength was almost doubled if 1% sodium caseinate was added to the milk samples. This was true for both treatments and also both transglutaminase incubation temperatures. However increases in gel strength in samples incubated at 37°C were very high compared to samples incubated at 55°C.

For samples incubated at both temperatures (37°C and 55°C) maximal gel strength was achieved when the transglutaminase concentration was 100 U/mL. The results also showed that for both temperatures, case 2, i.e., the non-inactivated samples, gave higher gel strength compared to case 1, especially when incubated at high enzyme concentrations (63.7g and 79.5g at week 1 and 2 respectively). The results also indicated that the storage time did affect gel strength in most all samples. Samples stored for two weeks showed lower gel strength than those stored for one week, except for case 2 incubated at 37°C, where samples that were incubated for the 2 week period appeared to have higher gel strengths than those incubated for a week. (Appendices D3 and D4).

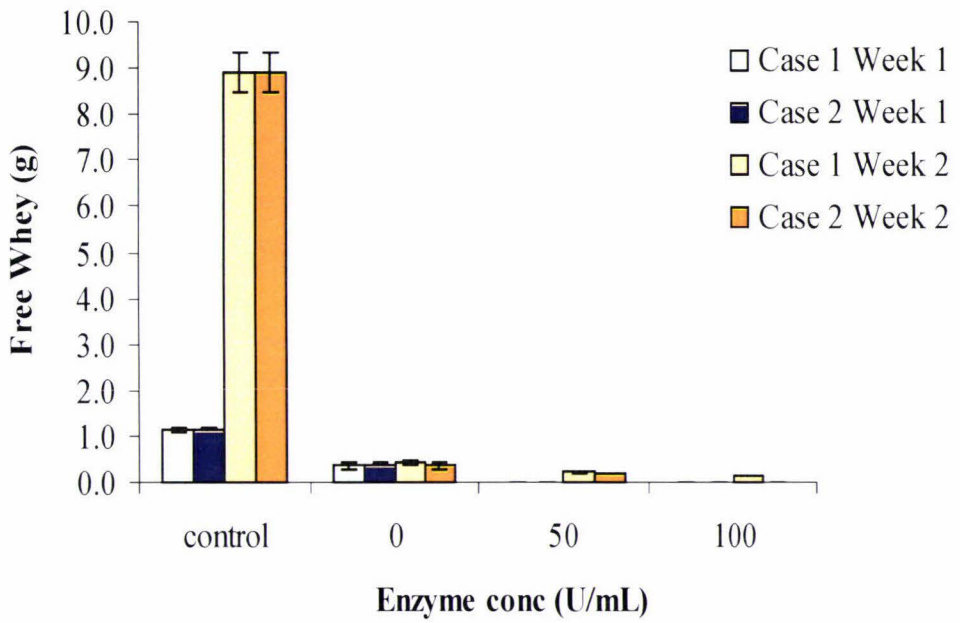

Figure 31: Free whey in acid milk gels treated with transglutaminase and sodium caseinate (1%) through time.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

Table VI: Differences in gel strength between Acid Milk Gels Incubated with Transglutaminase at different temperature, concentrations and storage time.

37°C	Case 1	Case 2	Case 1	Case 2
U/mL	Week 1	Week 1	Week 2	Week 2
control	28.4	28.4	22.5	28.4
0	49.9	49.9	51.4	51.4
50	47.9	58.7	46.5	57.1
100	59.1	63.7	55.2	79.5

55°C	Case 1	Case 2	Case 1	Case 2
U/mL	Week 1	Week 1	Week 2	Week 2
control	30.8	30.8	22.5	22.5
0	40.7	40.7	30.3	35.8
50	38.3	45.1	31.5	31.6
100	39.8	47.4	31.5	34.3

6.2.5 Shelf Life Behavior of Acid Milk Gels Treated with Transglutaminase, Sodium Caseinate and Milk Protein Isolate

In this experiment the effect of adding both sodium caseinate and milk protein isolate (1% NaCTMP) to a yogurt system was studied. A similar experimental design to that which was used in 5.2.4 was used for this experiment as well. Figure 32 shows the results of free whey in samples incubated at 37°C, and indicaties that for case 1 (enzyme inactivated samples) the addition of NacTMP had no effect on reducing the whey loss in the protein fortified controls compared to the non-fortified controls. In fact whey losses were higher (1,93 g) in the week old, enzyme inactivated, protein fortified controls compared to the non-fortified controls (1.15 g). However, the addition of transglutaminase significantly reduced this parameter 0.03g at 50 U/mL and 0g when using 100 U/mL. Case 2 seemed to respond better to whey reduction as Figure 32 shows. Storage time only appeared to affect the non-fortified yogurt controls. Whey losses jumped to 8.92, an almost 900% increase, in the extra week of storage. Samples with NacTMP showed no syneresis at all. However, samples with transglutaminase showed very little free whey compared to protein fortified controls that had been samples stored for one week (Figure 32). Samples incubated at 55°C and stored for one week showed a whey loss reduction of 86% for case 1 and 93% in

case 2 when using 100 U/mL of transglutaminase compared to the control sample (plain yogurt). The addition of NaCTMP with no transglutaminase reduced syneresis by 9.5% compared to the control (plain yogurt samples). This indicates that for this experiments even though the addition of NacTMP can reduce free whey, the major reductions were due to the addition of transglutaminase (Appendix D5-D6).

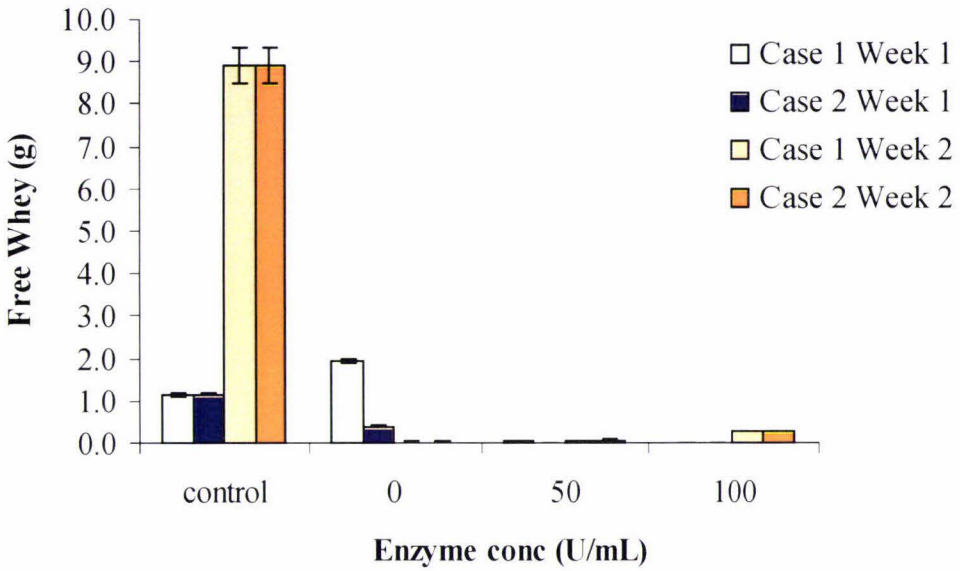

Figure 32: Free whey in acid milk gels treated with transglutaminase, sodium caseinate and milk protein isolate (1%) through time.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

Figure 33 shows the gel strength results over the 2 week storage time and between treatments. The experiments indicated that the non-fortified controls had significantly lower gel strengths than the protein fortified control samples. Gel strength of the protein fortified samples increased in an almost linear manner with increasing enzyme concentration. The one and two week old sample that had been treated with 100 U/mL of transglutaminase were almost double that of the non-protein fortified controls. After one week of storage gel strength increased notably specially when using 100 U/mL (72.35g and 71.30g) for case 1 and case 2 respectively when incubated at 37°C, while the control sample (plain yogurt) showed gel strengths of

28.4g and 43.80g for yogurts with NacTMP but no enzyme. However, week 2 samples showed lower results compared to week 1. The same linear trend of increasing gel strength with increasing enzyme concentration was evident in the two week old samples, though the gel strength was slightly less than the corresponding week old samples. Maximum gel strength for the 2 week old samples was also shown at an enzyme concentration of 100 U/mL, which was 56.47g and 57.18g for case 1 and case 2 respectively compared to 35.71g in samples with no enzyme and 22.45g in plain yogurt. Samples incubated at 55°C showed an increment of 34% compared to control sample for case 1 and 14% for case 2 in the first week, however notable increments were found after the second week (Appendix D7-D8). The whey losses and in particular gel strength results for samples fortified with milk protein isolate were quite different to those that had been fortified with sodium caseinate. In the case of the milk protein isolate fortified yogurt gel strength increase markedly with increasing enzyme concentration. This trend was nowhere near as marked for the sodium caseinate fortified yogurts. It would appear that the type of gel network that was formed in the yogurt system appeared to depend on the type of protein that was used to fortify the milk and this in turn affected the whey losses and gel strength of the resulting yogurts.

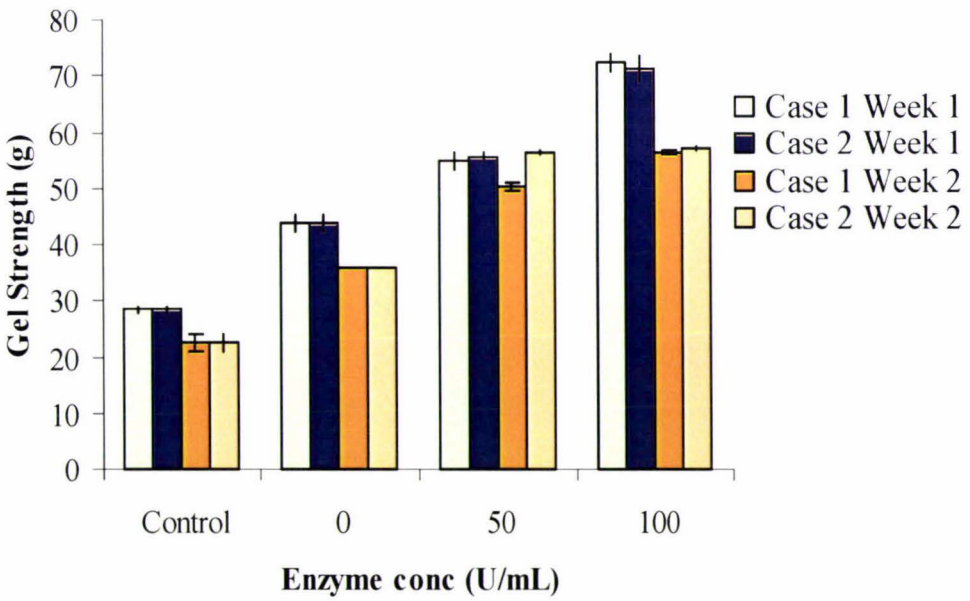

Figure 33: Gel strength in acid milk gels treated with transglutaminase, sodium caseinate and milk protein isolate.
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h) followed by inactivation (90°C for 2 min). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

6.2.6 Effect of Transglutaminase in Petit Suisse

Petit Suisse is a fully recombined yogurt with high fat and protein contents (Chapter III, section 3.2.8). Figure 34 shows the behaviour of Petit Suisse treated with transglutaminase at concentrations of 0, 50 and 100 U/mL and incubated at 37°C for 2 h followed by non-inactivation (case 2) and inactivation (case 1) at 90°C for 2 min. No significant differences between gel strength and enzyme concentration were observed at low enzyme concentrations. However at 100 U/mL, the gel strength reached a peak of approximately 250 g (five times higher than that of the control). The non-inactivated samples had significantly higher gel strengths than the inactivated samples when treated with transglutaminase at 100 U/mL. The experiment showed that the gel strength of Petit Suisse could be increased five-fold as long as sufficient enzyme was added to the system.

Figure 26 also shows the relationship between the shelf life and the gel strength in samples incubated with the enzyme. At low concentrations of enzyme, there were no significant differences in gel strength between day 1 and day 15 samples that had been stored at 5°C. Whereas the sample treated with enzyme at 100 U/mL showed a significant difference with time, falling by 50 g over the 15-day storage period (Appendix E).

6.3 Discussion

In this chapter the effect of adding transglutaminase to increasingly complex systems was explored. In the first experiment a very simple yogurt system consisting of skim milk, a starter culture and added transglutaminase was studied (Figure 25). The experiment showed that there was a concave relationship between whey loss and transglutaminase concentration, i.e. that some optimum amount of enzyme was necessary to minimise whey loss and that at concentrations on either side of the optimum concentration whey losses increased. This would suggest that an optimum number of cross-links are necessary before a suitable gel structure can be formed to contain the free whey. At enzyme concentrations below the critical optimum insufficient cross-links were formed and so whey losses occurred, and that the number of cross-links depended on enzyme concentration. At enzyme concentrations above this critical level too many cross-links are formed and as a consequence the gel structure shrinks with a consequential increase in the amount of whey that was lost.

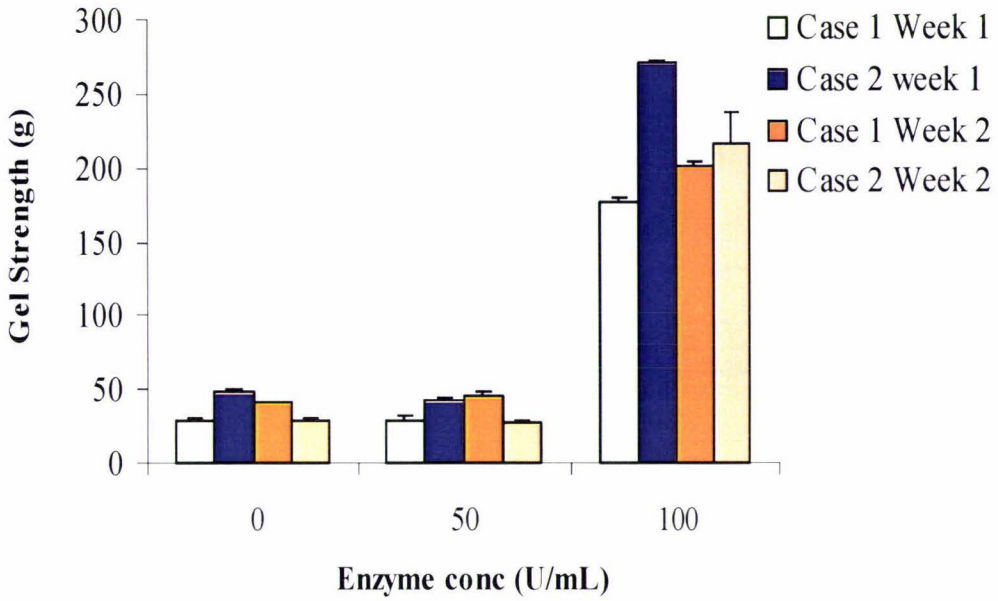

Figure 34: Gel strength in Petit Suisse treated with transglutaminase (0, 50, 100 U/mL).
Case 1: Acid milk gels treated with transglutaminase (37°C for 2 h), followed by inactivation (90°C for 2 min.). **Case 2**: Acid milk gels treated with transglutaminase (37°C for 2 h) together with starter inoculation and without inactivation.

Confocal microscopy would have been an ideal tool to follow the gel structure with varying levels of transglutaminase, but this technique was not used in this study. It is something that should be carried out in future experiments.

This first experiment also showed that whey losses were higher in samples of yogurt that had had the transglutaminase inactivated prior to the addition of the starter culture. These differences were evident for all enzyme concentrations. One is tempted to ascribe the differences in whey loss to the fact that the non-inactivated samples still contained active enzyme throughout the storage period and that this continued enzyme activity over the storage period was responsible for the differences in whey loss that was observed between the two treatments (Appendix C4). However, the fact that the trend was also evident in the respective controls suggests that the added heat treatment that was needed to inactivate the transglutaminase may have affected the water holding properties of the proteins and thus contributed to higher whey losses from the inactivated samples compared to the non-inactivated

samples. Clearly transglutaminase had some effect on whey loss, but further research is required to elucidate the contributions of the two mechanisms.

These results coincide with those obtained by Imm et al. (2000). In their study on the gelation and water binding properties of transglutaminase-treated skim milk powder, they showed that skim milk gels resulting from transglutaminase cross-linking showed a finer protein network with thin strands, compared with untreated skim milk gels. Also pilot plant studies done on the effect of enzymatic cross-linking in yogurt by Lorenzen and Schlimme (1997) (same as our case 1) indicated that syneresis was reduced with increasing enzyme concentration.

Dannenberg and Kessler (1988) have explained in detail why syneresis occurs. According to them syneresis occurs as a consequence of changes in the structure of the casein gel. These can occur even after the network has been completely formed. Attractive forces between individual casein particles or clustered micelles can lead to additional intermolecular bond rearrangements and, therefore, to a contraction of the gel and the expulsion of water. This phenomenon, called syneresis, is thus caused by a spontaneous release of water from the gel accompanied by a reduction in volume. It is brought about by changes in temperature and pH and by mechanical factors such as vibration. There is a relationship between the denaturation of β-lactoglobulin and the susceptibility of the yogurt gel to whey separation. An increase in the degree of β-lactoglobulin denaturation leads to a reduced susceptibility to syneresis.

The results obtained in this experiment suggested that the introduction of new cross-links by transglutaminase led to beneficial improvement in the gelation properties of milk protein gels. As the gel firmness increased, nearly doubling in the case of the non-inactivated samples treated with 100 and 200 U/mL compared to the control samples. It is suspected that this substantial increase in gel strength was brought about by the incorporation of new cross-links, which might have brought about the improvement in the water holding capacities and also the gelation properties of the resulting gels. The increased water holding capacity is related to the integrity of the protein matrix and is therefore related to syneresis and firmness.

Lorenzen (2000), in his studies on the techno-functional properties of transglutaminase-treated milk proteins, obtained very similar results to those obtained in this experiment. Lower whey syneresis and higher gel strength (Figures 25 and 26), together with milder taste and a smoother, drier, slightly whiter surface than untreated products were the main characteristics of the gels prepared. While

Faergemand (1998) has indicated that transglutaminase loses activity at pH 5 and is completely inactive at pH 4.3. However, in this research, it was found that, when the enzyme was left active during storage, a slight increase in gel strength and a reduction in syneresis occurred, indicating that the transglutaminase was not completely inactive, as suggested by Faergemand (1998). This might have been because it takessome time for the pH to drop, around 4–5 h (Michelle Harnett, personal communication).
Our study indicated that transglutaminase did not affect the samples pH during (Appendix C1-C3).

In the second experiment in this study the complexity of the acid milk gel was increased by incorporating 0.5% of sodium caseinate to the raw materials. This had a dramatic effect on the whey holding properties of the resulting gel. Whey losses were almost eliminated in samples that were treated with transglutaminase. The non-inactivated samples showed almost no whey loss for all transglutaminase treatments (Figure 27). Whereas the inactivated samples showed a minimal whey loss at transglutaminase concentration between 50 – 100 U/mL. The respective controls showed significant whey losses and the non-inactivated control lost significantly less whey than the inactivated control. The differences were significantly larger than was obtained for the simple yogurt system. This suggests that it was the caseins that were responsible for the high whey losses in both controls and that the extra heat treatment given to inactivated controls had some bearing on the higher whey losses from this treatment compared to the non-inactivated control. this total reduction could be found even at low enzyme concentrations of 10 U/mL for case 2. Two potential reasons could explain the large differences that were observed between the controls and the enzyme treated samples. The first reason rests on the assumption that the increased amounts of casein led to an increase in protein-water and protein-protein interactions, which in turn reduced the amount of wheying off from the system. As Rohm and Kneifel (1993) indicated in their study on physical properties of set yogurts that were enriched with different substrates that the addition of sodium caseinate to skim milk produced yogurts with least whey seepage. The second reason is that sodium caseinate could be a very good substrate for transglutaminase. This is quite apparent if Figures 25 and figure 27 are compared as they show a clear transglutaminase-sodium caseinate response. This last reason was proven by

Lorenzen, (2000) who indicated that sodium caseinate is an effective substrate for transglutaminase, whereas cross-linking of native whey protein is limited. However, polymerisation increases linearly when the degree of whey protein denaturation is increased by heat.

Nio et al. (1986) found that protein solutions of high concentration formed firm three-dimensional network gels that could be attributed to the production of casein oligomers as a consequence of transglutaminase cross-linking. This postulation was supported by the findings that the gelation of native α_{s1}-casein was dependent on the amount of transglutaminase.

In the third study in this series the type of protein was changed and 0.5% milk protein isolate was added in place of sodium caseinate (see Figure 29). This protein has less casein than sodium caseinate and more of the whey proteins. The trend in whey losses was quite different and was more like ordinary skim milk than the results that were obtained by adding sodium caseinate. In the case of added sodium caseinate whey losses were reduced to almost zero when sodium caseinate was added to the yogurt system. This was not the case for the milk protein isolate system. Whey losses followed a concave relationship with increasing enzyme concentration and the optimum enzyme level proved to be 100 U/mL for both the inactivated and the non-inactivated treatments. Whey losses increased dramatically when 200 U/mL was added to the inactivated sample, though no such increase was evident for the non-inactivated sample. The whey losses from this system were much lower than they were for the system with just skim milk. Clearly the added protein had some effect on the protein-water interactions and as a consequence reduced whey loss. The three experiments suggest that the caseinates are the critical proteins that are responsible for gel formation in yogurt systems and in particular for yogurt systems with added transglutaminase. It would seem that some minimum casein level is necessary before whey loss is almost suppressed. There didn't appear to be enough casein in the 0.5% of added milk protein isolate to completely suppress whey loss

The addition of 1% sodium caseinate was evaluated in a fourth experiment and the whey losses were compared with a set of controls that had no added protein. Whey losses were almost zero even for the 1% added sodium caseinate controls and transglutaminase appeared to have little effect on the amount of whey that was lost from this enriched caseinate system. The differences were most apparent in the

samples that had been stored for 2 weeks where the whey losses from the non-fortified controls were almost 900% higher than they had been after 1 weeks storage.. Inactivation of the enzyme prior to culture addition had no noticeable effect on the whey losses when compared with the same non-inactivated samples. These results suggest that if enough casein can be added to a yogurt system then whey losses can be minimized and that the benefits of adding transglutaminase are marginal when compared to just adding 1% sodium caseinate. When samples were incubated at 55°C, the whey reduction or syneresis depended closely on the transglutaminase concentrations. However, these results were less successful than the ones found when incubated at 37°C (Appendices D1 and D2).

However, gel strength did increase with increasing concentration of transglutaminase suggesting that the transglutaminase is needed, particularly at the higher concentrations (100 U/mL) to achieve significant improvements in gel strength. The results support the view that whey losses are dependent on 2 mechanisms: protein-water interactions and formation of an adequate gel structure to withstand deformation forces. Gel strength on the hand appears to be dependent on protein concentration and transglutaminase cross-linking

Gel strength in acid milk gels incubated with transglutaminase and 1.0% sodium caseinate through time showed that samples incubated at 37°C had a gel strength that was almost double that of the samples incubated at 55°C, especially case 2 samples which showed the highest gel strength when treated with 100 U/mL of transglutaminase. The shelf life analysis indicated a significant increase in gel firmness after the second week compared to the first week's results. Firmness clearly increased with time and also depended on the temperature at which the samples were incubated with transglutaminase. The samples stored at 37°C had a gel strength 20 grams force higher than the same treatment samples incubated at 55°C. Moreover, the 37°C samples' gel strength was 50 grams force higher than the samples that contained neither added neither sodium caseinate nor transglutaminase. These results would suggest that some additional cross-linking took place during the period of storage and that this was responsible for the increased gel strength of the stored samples. A minimum casein concentration also seems to be essential to ensure that the yogurt develops adequate gel strength. Clearly, the incubation temperature had some effect on cross-link formation and an incubation temperature of 37°C appears to be a more appropriate temperature for carrying out the transglutaminase reactions

than 55°C. In retrospect the gel samples should have been subjected to confocal microscopy to evaluate the effects of protein concentration, incubation period and transglutaminase concentration on gel structure as this would have given a greater insight into what was happening at the molecular level as a consequence of changes to the above variables.

A last experiment in the series examined what effect a combination of added proteins would have on the properties of the yogurt (acid gel). In this experiment 1% of NacTMP was added to the yogurt system. The results (Figure 32) were very similar to those that had been obtained with just 1 % sodium caseinate (Figure 31). The loss of whey followed a very similar pattern in both treatments. However, the whey losses for the protein combination system were slightly less for the protein combination than they were for just caseinate alone when the transglutaminase concentration was equal to greater than 50 U/mL. Clearly in this protein combination system there was enough of the critical milk proteins to form a gel that would contain the free whey. This would suggest that it was not just the casein that was responsible for the formation of the gel network, but that there were other proteins involved and these could have been the whey proteins which are in significantly higher concentration in the milk protein isolate than in sodium caseinate.

The gel strength of this protein combination gradually increased with increasing transglutaminase concentration (Figure 33). The highest firmness was evident in samples that had been incubated at 37°C with 100 U/mL. This occurred in the one week old samples. Storage time drastically reduced product firmness, as the 100 U/mL samples were 60 grams force less strong after two weeks storage compared to the one week old samples. The results for the combined protein system were different to the 1% sodium caseinate samples, which showed no drastic drop in firmness with storage time. This would suggest that relative concentrations of the major milk proteins in the acid gel had some bearing on the response of the gel to storage. It would appear that the combined gel possibly dissociated in some way over time whereas this was not the case for the casein gel did not. Or alternatively, and this is probably the main reason for the behaviour of the respective systems, the two systems produced mixed gels and the characteristics of portions of the gel behaved differently over time. The gel with a greater proportion of caseinate was more stable than the combined protein gel. In spite of these differences in behaviour it I clear that milk

protein isolate is a candidate for protein enrichment of yogurt systems as the protein produced gels that weeped less whey and had higher gel strength than yogurt made without protein enhancement.

Petit Suisse is a high fat, high protein acid gel. No whey loss occurred in any of the treatments. It is thought that the high protein of the system had a major bearing on the fact that no way loss occurred in much the same way as the yogurt system with 1% added sodium caseinate lost little weight. All the results from this series of experiments point to the need for a high protein content in the acid gel if whey losses are to be avoided. Transglutaminase addition had little effect on whey loss simply because of the high protein content of the system. However, gel strength did increase with increasing transglutaminase concentration suggesting that increased cross-link formation had some bearing on the gel strength of this product. The potential reason for such great gel strength could be due to the presence of the protein layer on the fat globule. If this was present the gel strength was high and if absent the gel strength was weak. This finding may explain why the presence of fat in dairy products affects texture either positively or negatively, and also why some dairy products derived from homogenised milk have different rheological properties from those made from non-homogenised milk. In effect, fat globules in milk or cream lose part of their membrane during homogenisation; the newly formed surfaces of the smaller fat globules adsorb casein particles or plasma proteins and the fat globules can then participate in acid or enzymatic coagulation processes (Buchheim, 1986; cited by Aguilera *et al.*, 1993).

Gel strength over time was significantly affected by the presence of transglutaminase and the effects were most obvious for samples treated with 100 U/mL of transglutaminase, particularly in the non-inactivated samples. The gel strength of the non-inactivated samples actually increased over the storage period and was significantly higher than the one week old samples from the same treatment. It would appear, therefore that enzyme activity continued over the storage period. This is something that did not happen in the sodium caseinate milk protein isolate samples and the difference in response could possibly be because there was surplus protein in the petit Suisse system to participate in further cross-linking, but was not so in the combined protein yogurt system. It could also be because additional lysine and glutamine residues were exposed on the milk globule surfaces and these were not available in the low fat yogurt system. However, further experimental work is needed

to elucidate the reasons for the increased development of gel strength over the two week storage period.

6.4 Conclusions

The addition of transglutaminase to yogurt resulted in a substantial reduction of free whey in the product. The reduction in whey loss was directly proportional to the concentration of transglutaminase up to a concentration of 100 U/mL. At concentrations in excess of 100 U/mL whey loss increased. The yogurt experiments demonstrated that transglutaminase would be a very effective additive for yogurt to prevent whey loss provided no more than 100 U/mL of transglutaminase was added to the product.

Whey losses were further reduced in the yogurt systems by not inactivating the transglutaminase after the incubation step. The experiment demonstrated that additional cross-linking over and above that, which occurred in the incubation step, was necessary to minimise whey loss.

Addition of sodium caseinate and milk protein isolate to yogurt resulted in a substantial reduction in the amount of whey that was lost from the yogurt at all concentrations of transglutaminase. The whey loss from the sodium caseinate and milk protein isolate enhanced products was significantly less than that from yogurts made with just milk. However, whey losses from their respective control samples varied. The experiment suggested that ordinary milk does not contain adequate concentrations of transglutaminase reactive proteins and that these proteins should be added to minimise the loss of whey from transglutaminase modified yogurts.

The gel strength of the yogurt was directly proportional to the amount of transglutaminase that was added to the yogurt system. Furthermore, inactivation of transglutaminase after the incubation stage resulted in a less strong gel for all transglutaminase concentrations than the non-inactivated enzyme samples. The experiments suggested that it is unnecessary to inactivate the enzyme after the incubation stage, as the gel strength of the product will continue to improve for some time during storage. However, experiments would need to be conducted to establish the optimum consumer acceptable gel strength so that the transglutaminase addition rates were optimised for both gel strength and whey loss.

Low variations in pH were found in all milk gel samples (Appendix C1, 2 and 3).

One percent of sodium caseinate and the combination of sodium caseinate with milk protein isolate reduced syneresis from yogurt notably compared to samples made with plain milk, and maintained this conditions throughout a two week storage period.. The addition of transglutaminase to the acid milk gels made with the above protein combination kept samples free from syneresis over the first week of storage, but small quantities of whey were lost after two weeks storage. This combination of proteins could b used to enhance the whey holding capacity of yogurt, but was not as effective as sodium caseinate alone (1%).

Experiments with a Petit Suisse system showed the same trends that were demonstrated for the yogurt system with a reduction in whey loss and increase in gel strength with increasing transglutaminase concentration up to a maximum of 100 U/mL.

Whey losses were substantially less from the Petit Suisse system than the yogurt system for all concentrations of transglutaminase suggesting that the increased solids and fat in Petit Suisse system had some major bearing on the resulting properties of the transglutaminase modified gel.

Gel strength of the Petit Suisse system containing active transglutaminase tended to improve with storage (2 weeks), provided the transglutaminase concentration was kept at 100 U/mL. At concentrations in excess of 100 U/mL gel strength dropped with increasing storage time.

Finally, this research confirms that transglutaminase can be used in milk products such as yogurts and could have a potential use on desserts, cheese etc. to improve the physical characteristics and to reduce the amount of dry matter.

CHAPTER VII

7.1 CONCLUSIONS

The research showed that milk proteins, and in particular β, κ-casein and β-lactoglobulin, were very good substrates for cross-linking by transglutaminase. Pre-heating the milk system prior to the addition of transglutaminase was shown to enhance milk protein cross-linking especially in Full Fat milk. Little cross-linking occurred until the Transglutaminase concentration reached 100 U/mL. The best enzyme incubation temperature was 37°C, and homogenization was an essential step when using milk with high fat content.

In most cases the addition of milk protein concentrates improved protein cross-linkage of most proteins when compared with samples with no added protein concentrate. This was demonstrated by a loss of monomeric protein bands and a corresponding accumulation of protein at the top of each lane. Sodium caseinate and protein isolate (TMP) proved to be the best proteins for enhancing transglutaminase mediated cross-linking of milk proteins.

Addition of transglutaminase to acid milk gels proved to be a very effective way of increasing gel strength, whilst at the same time minimizing syneresis. The addition of either 0.5% sodium caseinate or 0.5% milk protein isolate to acid gels greatly improved their gel strength and whey holding properties. The addition of 1% of NaCNTMP (50/50 mixture of sodium caseinate and total milk proteinate) dramatically improved the whey holding and gel strength properties of the gels over a two week storage period at 5°C compared to plain yogurt. The addition of transglutaminase to the NaCNTMP marginally improved the whey holding and gel strength properties of the gels over the two week period when compared with the NaCNTMP controls. This was especially case if the transglutaminase had not been inactivated after the Transglutaminase incubation period. Lastly Transglutaminase at 100 U/mL in a Petit Suisse gel system significantly improved the gel strength up to five hundred times that of the control samples and maintained its texture throughout the two week storage period. It would appear therefore from this study that Transglutaminase should be added to acid milk gels to minimize whey loss and maintain or improve gel strength. However, the costs of adding the transglutaminase would need to be balanced against the improved gel strength and

whey holding properties. Moreover, sensory trials would need to be conducted to assess the impact of Transglutaminase on the sensory properties of acid gels.

7.2 GENERAL RECOMMENDATIONS

The improvements that were obtained in the physical properties of acid gels as a consequence of the addition of transglutaminase suggest that this enzyme should be added to all acid milk gels so long as the cost of using the enzyme is not excessive. However, before this general recommendation is implemented further work is needed to assess the impact of using this enzyme at a concentration between 50–100 U/mL should be assessed.

It would appear that the milk proteins, and in particular the whey proteins, should be denatured prior to the addition of the enzyme by an appropriate heat treatment. In this study the milk was heated for 10 minutes at 90°C and then cooled to 37°C before the enzyme was added. However, further work is needed to determine whether the heat treatment is resulting in aggregation of the whey proteins or whether the heat treatment actually sensitises the whey proteins to enzyme cross-linkage, especially when the enzyme concentration is 100 U/mL. Reduced SDS PAGE should be conducted on each batch to determine whether disulphide bridging or transglutaminase mediated cross-linking was responsible for the loss of monomeric forms of the whey proteins that were observed in this study. Proteolysis of the acid milk gels followed by HPLC would also provide proof of the formation of ε–γ (glutamyl) lysine bonds formed as a consequence of transglutaminase activity.

The study suggested that the milk gels should be incubated at 37°C before the addition of transglutaminase was added to maximise cross-linking. However, as only two temperatures were used in this study it is suggested that further work should be carried out to optimise the transglutaminase incubation temperature and time.

The study also suggested that cross-linking was maximised if the Transglutaminase was not inactivated prior to the addition of the starter. This should be confirmed in a further study.

The addition of protein concentrates, and in particular sodium caseinate and milk protein isolate, to the acid milk gels dramatically improved the amount of proteins that were cross-linked and the storage capabilities of the gels. However, marked differences between the proteins that were cross-linked were found between the

various added protein concentrates. The work suggested that the composition of the added protein concentrate was responsible for the observed differences. This would need to be confirmed in further work. The structure of gels should be followed by use of electron and confocal microscopy. Reduced SDS PAGE should also be conducted to see whether the cross-links were caused by disulphide bridging or by transglutaminase cross-linking. The amount of added protein also appeared to be significant in determining the amount of cross-linking and the physical properties of the gels and this needs to be followed up to assess what effect protein concentration has on the microscopical properties of the gels as well as the nature of the cross-links.

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APPENDIX A

A1 Total Solids in Trim Milk (37°C)

Units/mL	Unheated Dish Weight	Dish + sample	Final Weight	Total Solid
Control	15.1808	17.4115	15.4587	12.457973
	23.1094	25.017	23.34	12.088488
1	16.2976	18.472	16.5984	13.833701
	23.2566	25.666	23.5564	12.442932
5	23.4118	25.6346	23.6875	12.403275
	23.4695	25.6931	23.7602	13.073394
10	22.6062	24.7282	22.8461	11.305372
	23.2469	25.5003	23.4899	10.783705
100	23.8003	25.9617	24.0438	11.265846
	16.0992	18.1777	16.3341	11.301419
	Heated			
Control	11.0396	13.1276	11.302	12.56705
	10.5442	12.6405	10.8	12.202452
1	10.8432	12.878	11.1059	12.91036
	11.0422	13.0852	11.2954	12.393539
5	16.197	18.1303	16.4478	12.972637
	10.9211	12.9743	11.1864	12.921294
10	22.6608	24.7308	22.9279	12.903382
	8.859	10.813	9.0969	12.175026
100	22.7963	24.9009	23.0589	12.47743
	22.5573	25.0219	22.8751	12.894587

A2 Total Solids in Full Fat Milk (37°C)

Units/mL	Unheated Dish Weight	Dish + sample	Final Weight	Total Solid
Control	24.0864	26.3656	24.4426	15.628291
	11.6838	13.6933	11.99	15.237621
1	16.1996	18.3618	16.5084	14.28175
	23.4329	25.4367	23.7277	14.712047
5	23.4957	25.5406	23.7934	14.558169
	10.9219	12.9386	11.2368	15.614618
10	9.3597	11.4267	9.6752	15.263667
	16.1962	18.3253	16.4987	14.207881
100	10.4932	12.5493	10.8	14.921453
	11.2819	13.3835	11.5963	14.96003
	Heated			
Control	10.722	12.8798	11.0875	16.938549
	10.8482	12.8928	11.1868	16.560696
1	11.0784	13.1292	11.4264	16.968988
	12.8956	15.2002	13.2243	14.262779
5	9.299	11.3206	9.6221	15.98239
	11.0091	13.0672	11.3162	14.92153
10	14.3835	16.4433	14.6562	13.239149
	16.2117	18.3299	16.4886	13.07242
100	9.3353	11.614	9.6852	15.355246
	10.5062	13.0983	10.9318	16.41912

A3 **Total Solids in Trim Milk (55°C)**

Units/mL	Dish Weight	Dish + sample	Final Weight	Total Solid
	Unheated			
Control	15.1808	17.4115	15.4325	11.283454
	23.1094	25.017	23.3305	11.59048
1	23.2566	25.666	23.5564	12.442932
	16.2976	18.472	16.5984	13.833701
5	23.0533	25.1308	23.2655	10.2142
	32.9647	36.1885	33.2923	10.161921
10	32.9654	34.4808	33.1295	10.828824
	23.3393	25.0752	23.5221	10.530561
100	24.0037	26.0117	24.2149	10.517928
	23.8315	25.9091	24.0505	10.541009
	Heated			
Control	15.3207	17.4333	15.5948	12.974534
	23.3797	25.4647	23.6335	12.172662
1	9.4382	11.429	9.6952	12.909383
	11.872	13.915	12.1254	12.403328
5	9.4434	11.4893	9.6859	11.852974
	22.346	24.5789	22.5698	10.02284
10	22.6608	24.7308	22.9279	12.903382
	8.859	10.813	9.0969	12.175026
100	22.7963	24.9009	23.0978	14.325763
	22.5573	25.0219	22.9083	14.241662

A4 **Total Solids in Full Fat Milk (55°C)**

Units/mL	Dish Weight	Dish + sample	Final Weight	Total Solid
	Unheated			
Control	8.3893	10.3832	8.6391	12.528211
	7.1951	9.2341	7.4498	12.491417
1	15.9108	18.0076	16.2103	14.28367
	8.3969	10.4065	8.6806	14.117237
5	9.1797	11.2563	9.4758	14.258885
	10.7865	12.8922	11.0899	14.40851
10	11.0367	13.3532	11.377	14.690265
	14.7655	16.8563	15.0754	14.822078
100	15.9036	18.0592	16.1861	13.1054
	7.8133	9.8303	8.0783	13.138324
	Heated			
Control				
1	11.0412	13.1296	11.3286	13.761731
	10.9929	13.169	11.2957	13.914802
5	23.4118	25.6346	23.7312	14.369264
	23.4695	25.6931	23.7602	13.073394
10	22.5825	24.6763	22.8963	14.987105
	24.0841	26.2285	24.3922	14.367655
100	22.7963	24.9009	23.1099	14.900694
	22.5573	25.0219	22.9083	14.241662

APPENDIX B

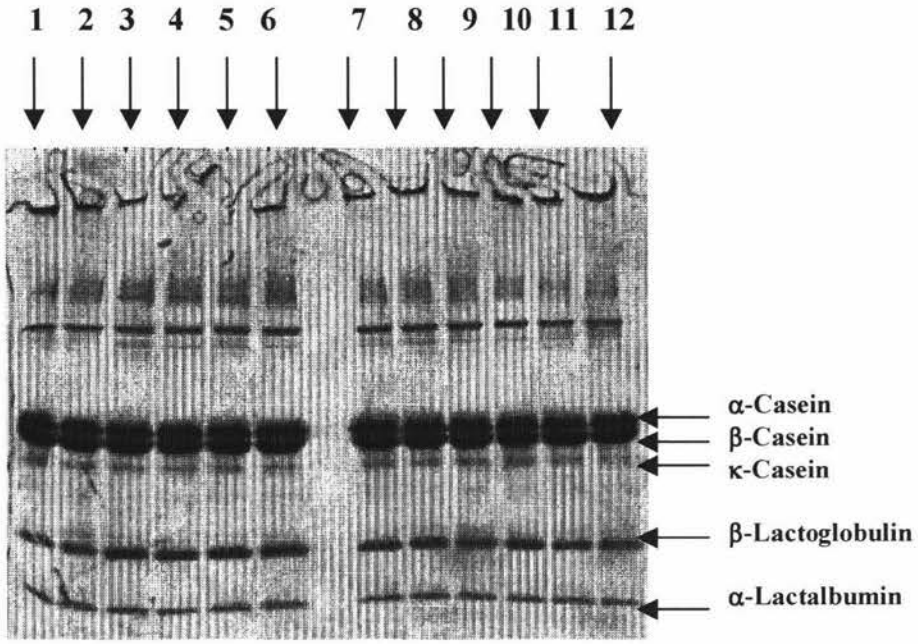
Concept: SDS is a negatively charged surface-active substance. It is used to disrupt the non-covalent bonds, through its ability to adsorb to hydrophobic and positively charged sites on proteins. Different proteins bind almost the same amount of SDS on a mass basis. Thus, once coated with SDS, the proteins can be separated by electrophoresis on the basis of the molecular size of the protein–SDS complexes.

Procedure: Samples were prepared by diluting the milk 40 times with SDS buffer (0.01 mol/dm^3 Tris-HCl, pH 8.0, 2.5% SDS, 0.01% bromophenol blue). When analysing for the contribution that disulphide bonds made to protein cross-linking, 2-mercaptoethanol was used as a reducing agent to rupture any existing disulphide bonds.

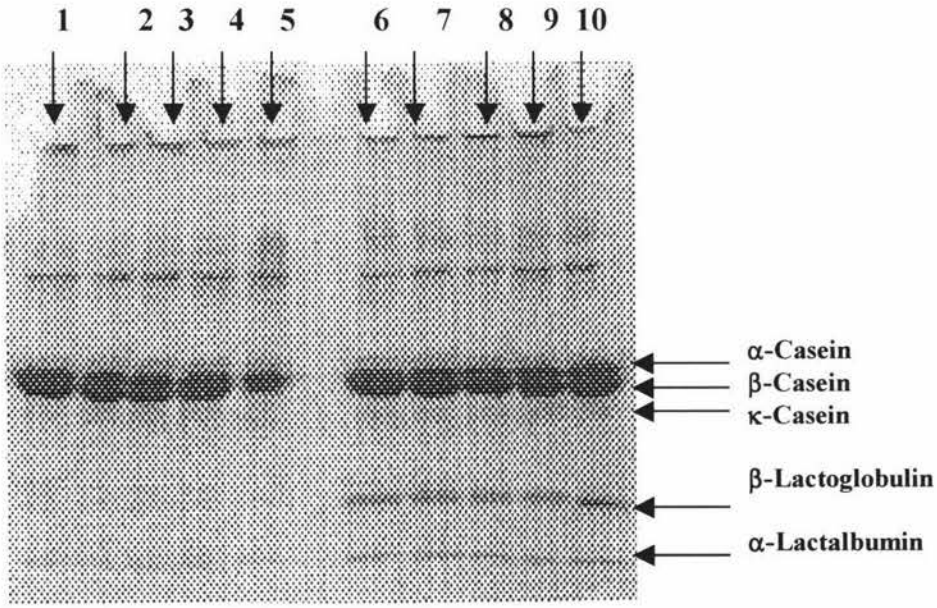
A tracking dye (bromophenol blue) was included to monitor the movement of proteins during electrophoresis. The tracking dye migrates at a faster rate than any of the proteins in the sample and indicates when electrophoresis should be terminated. A 200volt potential difference between the electrodes was used for all electrophoresis runs.

Following electrophoresis, the separated proteins were visualised by staining the gel for 1 h in a Coomassie Blue R-250 solution or Amido Black 10B. The dye molecules were dissolved in an aqueous mix of isopropanol and acetic acid. Isopropanol has a precipitating effect on the protein–dye complex, which reduces leaching of the proteins from the gel. After staining, the entire gel was coloured. To leach out excess dye, the gel was destained with an acetic acid/isopropanol solution. The rate of destaining depended on whether agitation was applied. Samples were analysed only once.

Once the gels were destained, densitometry was performed by analysing scanned images of the gels using Image Quant and Excel software.



SDS-PAGE gel of Trim™ milk treated with transglutaminase (55°C for 2 h). Samples 1–6 were pre-unheated, and samples 7–12 were pre-heated at 90°C for 10 min. **1 and 2**: control unheated; **3**: 1 U/mL; **4**: 5 U/mL; **5**: 10 U/mL; **6**: 100 U/mL; **7**: 1 U/mL; **8**: 5 U/mL; **9**: 10 U/mL; **10**: 100 U/mL; **11 and 12:** control (heated).



SDS-PAGE gel of Full Fat non-homogenized milk treated with transglutaminase and 0.5% MPC. Samples 1–6 were incubated at 37°C and samples 7–12 were incubated at 55°C (2h). **1** : control unheated; **2**: 1 U/mL; **3**: 5 U/mL; **4**: 10 U/mL; **5**: 100 U/mL; **6**: 1 U/mL; **7**: 5 U/mL; **8**: 10 U/mL; **9**: 100 U/mL; **11:** control (heated).

APPENDIX C

Measurement of pH in yogurt with Transglutaminase

Case 1

C1 pH

U/mL	S 1	S 2	S 3	Average 1
0	4.4	4.4	4.4	**4.4**
10	4.4	4.3	4.3	**4.3**
50	4.3	4.2	4.2	**4.2**
100	4.2	4.2	4.1	**4.2**
200	4.1	4.1	4.1	**4.1**

Case 2

pH

U/mL	S1	S 2	S 3	Average 2
0	4.5	4.5	4.5	**4.5**
10	4.5	4.5	4.51	**4.5**
50	4.6	4.6	4.7	**4.6**
100	4.6	4.6	4.5	**4.6**
200	4.5	4.5	4.5	**4.5**

Measurement of pH in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

C2 pH

U/mL	S 1	S 2	S 3	Average 1
0	4.3	4.2	4.3	**4.30**
10	4.4	4.3	4.3	**4.3**
50	4.4	4.4	4.3	**4.4**
100	4.2	4.3	4.3	**4.3**
200	4.4	4.3	4.3	**4.3**

Case 2

pH

U/mL	S1	S 2	S 3	Average 2
0	4.3	4.3	4.3	**4.3**
10	4.3	4.3	4.3	**4.3**
50	4.3	4.4	4.3	**4.3**
100	4.3	4.2	4.3	**4.3**
200	4.2	4.2	4.3	**4.2**

Measurement of pH in yoghurt with Milk Protein Isolate and Transglutaminase

Case 1

C3 pH

U/mL	S 1	S 2	S 3	Average 1
0	4.2	4.2	4.2	**4.20**
10	4.2	4.2	4.2	**4.2**
50	4.3	4.2	4.2	**4.2**
100	4.3	4.3	4.2	**4.3**
200	4.2	4.3	4.2	**4.2**

Case 2

pH

U/mL	S1	S 2	S 3	Average 2
0	4.2	4.2	4.2	**4.2**
10	4.1	4.2	4.2	**4.2**
50	4.2	4.1	4.1	**4.2**
100	4.1	4.1	4.1	**4.1**
200	4	4.1	4	**4.1**

Measurement of Free Whey in yogurt with Transglutaminase

Case 1

C4 **Whey Drain (g)**

U/mL	S 1	S 2	S 3	Average 1
0	2.2	2	2.3	**2.16**
10	0.3	1	0	**0.86**
50	3.1	2.5	2.8	**2.8**
100	1	1.3	1.3	**1.2**
200	0.6	0.6	1.5	**0.9**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
0	1.8	1.7	1.6	**1.7**
10	1	0.4	0.9	**0.76**
50	0	0.4	0.2	**0.2**
100	0	0	0.2	**0.06**
200	0.3	0.4	0.3	**0.33**

Measurement of Free Whey in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

C5 **Whey Drain (g)**

U/mL	S 1	S 2	S 3	Average 1
0	4.51	4.84	7.1	**5.48**
10	0	0.55	0.6	**0.38**
50	0	0	0.12	**0.04**
100	0	0.1	0.08	**0.06**
200	0.1	0.2	0.32	**0.21**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
0	0	0	0	**0**
10	0	0	0.13	**0.04**
50	0	0	0	**0**
100	0	0.06	0	**0.02**
200	0	0	0	**0**

Measurement of Free Whey in yoghurt with Milk Protein Isolate and Transglutaminase

Case 1

C6 **Whey Drain (g)**

U/mL	S 1	S 2	S 3	Average 1
0	0.42	0.48	0.62	**0.51**
10	0.3	0.3	0.45	**0.35**
50	0.32	0	0.4	**0.24**
100	0	0	0	**0**
200	0.9	1	0.7	**0.87**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Avg 2
0	0.6	0.4	0.61	**0.54**
10	0.2	0.35	0.23	**0.26**
50	0	0	0.15	**0.05**
100	0	0	0	**0**
200	0	0	0	**0**

Measurement of Gel Strength in yogurt with Transglutaminase

Case 1

C7 **Force (g)**

U/mL	S 1	S 2	S 3	Average 1
0	29.91	28.86	31	**29.9125**
10	31.31	33.01	30.9	**31.756**
50	29.13	28.25	29.6	**28.977**
100	38.64	40.63	39.9	**39.721**
200	27	25.2	28.5	**26.905**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
0	31.64	31.373	28.85	**30.619**
10	30.45	30.976	28.74	**30.054**
50	33.7	35.687	35.31	**34.9**
100	53.88	55.029	55.86	**54.922**
200	51.22	54.291	52.3	**52.601**

Measurement of Gel Strength in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

C8 **Force (g)**

U/mL	S 1	S 2	S 3	Average 1
0	54.85	56.85	54.10	**55.26**
10	48.55	54.69	51.20	**51.48**
50	58.12	58.20	58.01	**58.11**
100	63.67	64.55	67.00	**65.07**
200	62.62	62.21	64.46	**63.09**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
0	64.17	62.90	60.84	**62.64**
10	67.41	65.78	67.44	**66.88**
50	64.93	67.22	67.90	**66.68**
100	69.32	78.10	71.59	**73.00**
200	76.71	77.07	78.51	**77.43**

Measurement of Gel Strenth in yoghurt with Milk Protein Isolate and Transglutaminase

Case 1

C9 **Force (g)**

U/mL	S 1	S 2	S 3	Average 1
0	72.88	70.59	72.96	**72.14**
10	77.97	67.92	72.29	**72.73**
50	90.19	92.95	96.93	**93.36**
100	92.69	91.80	89.87	**91.45**
200	76.84	77.70	76.61	**77.05**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
0	76.07	75.23	75.67	**75.66**
10	74.08	77.13	75.89	**75.70**
50	87.43	83.34	87.73	**86.17**
100	96.20	106.37	92.92	**98.50**
200	83.13	80.19	83.78	**82.37**

Case 1: Yoguth treated with Transglutaminase (37°C 2h) previously inoculation
Case 2: Yogurth treated with Transglutaminase (37°C 2h) together with inoculation

APPENDIX D

37°C (concentrations: 0, 50, 100 U/mL)

Week 1

Measurement of Free Whey in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

D1 **Whey Drain (g)**

U/mL	S 1	S 2	S 3	Average 1
control	1.2	1.14	1.1	**1.15**
0	0.3	0.38	0.4	**0.36**
50	0	0	0	**0**
100	0	0	0	**0**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
control	1.2	1.14	1.1	**1.15**
0	0.3	0.38	0.4	**0.36**
50	0	0	0	**0**
100	0	0	0	**0**

Week 2

Measurement of Free Whey in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

Whey Drain (g)

U/mL	S 1	S 2	S 3	Average 1
control	8.48	9.36	8.92	**8.92**
0	0.4	0.4	0.5	**0.43**
50	0.21	0.24	0.21	**0.22**
100	0.12	0.14	0.13	**0.13**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
control	8.48	9.36	8.92	**8.92**
0	0.4	0.4	0.5	**0.36**
50	0.18	0.2	0.2	**0.19**
100	0	0	0	**0**

55°C (concentrations: 0, 50, 100 U/mL)

Week 1

Measurement of Free Whey in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

D2 **Whey Drain (g)**

U/mL	S 1	S 2	S 3	Average 1
control	7.62	6.96	6.21	**6.93**
0	6.08	6.18	6.05	**6.10**
50	0	0.52	0	**0.17**
100	0	0	0.12	**0.04**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
control	7.62	6.96	6.21	**6.93**
0	2.18	2.34	2.86	**2.46**
50	1.02	1.46	1.48	**1.32**
100	0	0	0	**0**

Week 2

Measurement of Free Whey in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

Whey Drain (g)

U/mL	S 1	S 2	S 3	Average 1
control	4.8	4.26	4.06	**4.37**
0	3.88	3.84	3.94	**3.89**
50	3.68	3.76	2.89	**3.44**
100	1.84	1.76	1.94	**1.85**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
control	4.8	4.26	4.06	**4.37**
0	1.34	1.28	1.46	**1.36**
50	0.52	0.48	0.4	**0.47**
100	0	0	0	**0**

37°C (concentrations: 0, 50, 100 U/mL)

Week 1

Measurement of Gel Strength in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

D3 **Force (g)**

U/mL	S 1	S 2	S 3	Average 1
Contro	27.8	29	28.4	**28.4**
0	50.90	48.90	49.90	**49.90**
50	46.39	49.50	47.95	**47.95**
100	59.44	60.41	57.42	**59.09**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
control	27.8	29	28.4	**28.4**
0	50.90	48.90	49.90	**49.90**
50	58.60	58.70	58.70	**58.67**
100	62.40	65.00	63.70	**63.70**

Week 2

Measurement of Gel Strength in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

Force (g)

U/mL	S 1	S 2	S 3	Average 1
Contro	20.85	24.06	22.5	**22.45**
0	51.52	51.29	51.41	**51.41**
50	44.45	48.48	46.46	**46.46**
100	54.73	55.70	55.22	**55.22**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
control	20.85	24.06	22.45	**28.4**
0	51.52	51.29	51.41	**51.41**
50	59.51	54.76	57.14	**57.14**
100	80.28	79.96	78.12	**79.45**

55°C (concentrations: 0, 50, 100 U /mL)

Week 1

Measurement of Gel Strength in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

D4 **Force (g)**

U/mL	S 1	S 2	S 3	Average 1
Contro	29.4	28.9	34	**30.76**
0	40.70	41.00	40.30	**40.67**
50	37.20	39.10	38.70	**38.33**
100	41.20	35.80	42.30	**39.77**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
control	29.4	28.9	34	**30.76**
0	40.70	41.00	40.30	**40.67**
50	44.20	45.10	45.90	**45.07**
100	47.22	48.10	46.90	**47.41**

Week 2

Measurement of Gel Strength in yoghurt with Sodium Caseinate and Transglutamianse

Case 1

Force (g)

U/mL	S 1	S 2	S 3	Average 1
Contro	22.18	22.54	22.6	**22.45**
0	28.29	31.66	30.91	**30.29**
50	30.83	32.44	31.23	**31.50**
100	29.81	33.31	31.35	**31.49**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
control	22.18	22.54	22.59	**22.45**
0	34.72	37.48	35.17	**35.79**
50	32.38	29.22	33.30	**31.63**
100	34.88	33.20	34.93	**34.34**

37°C (concentrations: 0, 50, 100 U/mL)

Week 1

Measurement of Free Whey in yoghurt with NACTMP and Transglutaminase

Case 1

D5 **Whey Drain (g)**

U/mL	S 1	S 2	S 3	Average 1
control	1.2	1.14	1.1	**1.15**
0	2.03	1.96	1.8	**1.93**
50	0	0.1	0	**0.03**
100	0	0	0	**0**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
control	1.2	1.14	1.1	**1.15**
0	2.03	1.96	1.8	**0.36**
50	0	0	0	**0**
100	0	0	0	**0**

Week 2

Measurement of Free Whey in yoghurt with NACTMP and Transglutaminase

Case 1

Whey Drain (g)

U/mL	S 1	S 2	S 3	Average 1
control	8.48	9.36	8.92	**8.92**
0	0	0	0	**0**
50	0	0.12	0	**0.04**
100	0.28	0.32	0.27	**0.29**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
control	8.48	9.36	8.92	**8.92**
0	0	0	0	**0.00**
50	0	0.2	0	**0.06**
100	0.26	0.3	0.29	**0.28**

55°C (concentrations: 0, 50, 100 U /mL)

Week 1

Measurement of Free Whey in yoghurt with NACTMP and Transglutaminase

Case 1

D6 **Whey Drain (g)**

U/mL	S 1	S 2	S 3	Average 1
control	15	15.03	15	**15.01**
0	13.36	13.2	13.2	**13.26**
50	3.21	3.34	3.13	**3.22**
100	2.17	2.05	2.11	**2.11**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
control	15	15.03	15.02	**15.01**
0	13.69	13.53	13.57	**13.59**
50	3.61	3.78	3.14	**3.51**
100	0.81	1.19	1.12	**1.04**

Week 2

Measurement of Free Whey in yoghurt with NACTMP and Transglutaminase

Case 1

Whey Drain (g)

U/mL	S 1	S 2	S 3	Average 1
control	15	15.03	15	**15.01**
0	4.72	4.38	5.02	**4.71**
50	13.04	13.06	11.7	**12.61**
100	16.08	17.02	17.1	**16.73**

Case 2

Whey Drain (g)

U/mL	S1	S 2	S 3	Average 2
control	15	15.03	15.02	**15.01**
0	0.46	0.6	0.9	**0.65**
50	10	12.6	13.22	**11.94**
100	0	0	0	**0**

37°C (concentrations: 0, 50, 100 U/mL)

Week 1

Measurement of Gel Strength in yoghurt with NACTMP and Transglutaminase

Case 1

D7 **Force (g)**

U/mL	S 1	S 2	S 3	Average 1
Contro	27.8	29	28.4	**28.4**
0	42.20	45.40	43.80	**43.80**
50	53.30	54.80	56.50	**54.87**
100	70.90	73.80	72.35	**72.35**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
control	27.8	29	28.4	**28.4**
0	42.20	45.40	43.80	**43.80**
50	56.60	55.50	54.95	**55.68**
100	73.70	68.90	71.30	**71.30**

Week 2

Measurement of Gel Strength in yoghurt with NACTMP and Transglutaminase

Case 1

Force (g)

U/mL	S 1	S 2	S 3	Average 1
Contro	20.85	24.06	22.5	**22.45**
0	35.78	35.63	35.71	**35.71**
50	51.05	49.42	50.23	**50.23**
100	56.88	56.39	56.14	**56.47**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
control	20.85	24.06	22.45	**22.45**
0	35.78	35.63	35.71	**35.71**
50	56.01	56.57	56.65	**56.41**
100	56.68	57.67	57.18	**57.18**

55°C (concentrations: 0, 50, 100 U /mL)

Week 1

Measurement of Gel Strength in yoghurt with NACTMP and Transglutaminase

Case 1

D8 **Force (g)**

U/mL	S 1	S 2	S 3	Average 1
Contro	29.4	28.9	30	**29.43**
0	32.70	33.10	31.00	**32.27**
50	36.60	33.00	35.60	**35.07**
100	40.00	39.10	39.20	**39.43**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
control	29.4	28.9	30	**29.43**
0	43.80	41.90	43.40	**43.03**
50	43.40	41.60	42.90	**42.63**
100	33.70	33.50	33.70	**33.63**

Week 2

Measurement of Gel Strength in yoghurt with NACTMP and Transglutaminase

Case 1

Force (g)

U/mL	S 1	S 2	S 3	Average 1
Contro	17.51	18.2	19.3	**18.32**
0	21.95	22.39	22.77	**22.37**
50	27.18	27.67	26.29	**27.05**
100	27.74	27.05	27.79	**27.53**

Case 2

Force (g)

U/mL	S1	S 2	S 3	Average 2
control	17.51	18.2	19.25	**18.32**
0	28.78	27.40		**28.09**
50	33.91	30.28	30.80	**31.66**
100	46.87	44.78	48.13	**46.59**

Case 1: Yoguth treated with Transglutaminase (37°C 2h) previously inoculation
Case 2: Yogurth treated with Transglutaminase (37°C 2h) together with inoculation

APPENDIX E

Gel strength in Petit Suisse

Force (g) [max peak]

U/mL	S 1	S 2	S 3	Case 1 Week 1	dst
0	29.032	31.006	28.957	**29.67**	**1.16**
50	29.612	27.876	29.688	**29.06**	**2.68**
100	173.979	179.346	176.696	**176.67**	**2.68**

Force (g) [max peak]

U/mL	S1	S 2	S 3	Case 2 Week 1	dst
0	50.433	47.451	48.867	**48.92**	**1.49**
50	43.662	42.652	42.434	**42.92**	**0.66**
100	271.623	273.42	270.273	**271.77**	**1.58**

Force (g) [max peak]

U/mL	S 1	S 2	S 3	Case 1 Week 2	dst
0	41.84	41.674	41.77	**41.76**	**0.08**
50	45.931	44.962	44.586	**45.16**	**3.28**
100	204.412	198.751	198.725	**200.63**	**3.28**

Force (g) [max peak]

U/mL	S1	S 2	S 3	Case 2 Week 2	dst
0	30.65	29.317	28.056	**29.34**	**1.30**
50	28.546	26.616	27.783	**27.65**	**0.97**
100	197.306	237.878	214.695	**216.63**	**20.35**

U/mL	Case 1 Week 1	Case 2 week 1	Case 1 Week 2	Case 2 Week 2	dst
0	29.67	48.92	41.76	29.34	**9.73**
50	29.06	42.92	45.16	27.65	**8.72**
100	176.67	271.77	200.63	216.63	**49.46**

case 1: Transglutaminase Inactive
case 2: Transglutaminase Active

APPENDIX F

Statistical Comparison of Shelf Life in Yogurts (Free Whey)

F1

Class Level Information

Class	Levels	Values
enzyme	3	0 50 100
day	2	1 15

Number of observations 208

NOTE: Due to missing values, only 192 observations can be used in this analysis.

The GLM Procedure

Dependent Variable: sqwhey

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	83.0816439	16.6163288	12.57	<.0001
Error	186	245.8641494	1.3218503		
Corrected Total	191	328.9457934			

R-Square	Coeff Var	Root MSE	sqwhey Mean
0.252569	82.43607	1.149717	1.394678

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enzyme	2	73.68262745	36.84131372	27.87	<.0001
day	1	3.39038176	3.39038176	2.56	0.1110
enzyme*day	2	6.00863472	3.00431736	2.27	0.1059

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enzyme	2	73.68262745	36.84131372	27.87	<.0001
day	1	6.31219142	6.31219142	4.78	0.0301
enzyme*day	2	6.00863472	3.00431736	2.27	0.1059

The GLM Procedure

Level of enzyme	Level of day	N	sqwhey Mean	sqwhey Std Dev
0	1	48	2.03963262	1.18290250
0	15	48	1.95595517	1.25616344
50	1	24	0.64486529	0.80243028

50	15	24	1.33840419	1.38247071
100	1	24	0.32304333	0.55424968
100	15	24	0.85993375	1.32044680

Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

enzyme	day	sqwhey LSMEAN	LSMEAN Number
0	1	2.03963262	1
0	15	1.95595517	2
50	1	0.64486529	3
50	15	1.33840419	4
100	1	0.32304333	5
100	15	0.85993375	6

Statistical Comparison of shelf life in yogurts (Gel Strength)

F2

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
enzyme	3	0 50 100
day	2	1 15

Number of observations 208

NOTE: Due to missing values, only 190 observations can be used in this analysis.

The GLM Procedure

Dependent Variable: sqforce

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	5	89.2678364	17.8535673	18.20	<.0001
Error	184	180.4809015	0.9808745		
Corrected Total	189	269.7487378			

R-Square	Coeff Var	Root MSE	sqforce Mean
0.330930	16.59321	0.990391	5.968654

Source	DF	Type I SS	Mean Square	F Value	Pr > F
enzyme	2	89.26783635	44.63391818	45.50	<.0001
day	1	0.00000000	0.00000000	0.00	1.0000
enzyme*day	2	0.00000000	0.00000000	0.00	1.0000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
enzyme	2	89.26783635	44.63391818	45.50	<.0001
day	1	0.00000000	0.00000000	0.00	1.0000
enzyme*day	2	0.00000000	0.00000000	0.00	1.0000

The GLM Procedure

Level of enzyme	Level of day	N	sqforce Mean	sqforce Std Dev
0	1	47	5.29881250	0.91534677
0	15	47	5.29881250	0.91534677
50	1	24	6.37888396	0.92539405
50	15	24	6.37888396	0.92539405
100	1	24	6.87019602	1.17958650
100	15	24	6.87019602	1.17958650

The GLM Procedure

Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

enzyme	day	sqforce LSMEAN	LSMEAN Number
0	1	5.29881250	1
0	15	5.29881250	2
50	1	6.37888396	3
50	15	6.37888396	4
100	1	6.87019602	5
100	15	6.87019602	6

Least Squares Means for effect enzyme*day
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sqforce

i/j	1	2	3	4	5	6
1		1.0000	0.0003	0.0003	<.0001	<.0001
2	1.0000		0.0003	0.0003	<.0001	<.0001
3	0.0003	0.0003		1.0000	0.5214	0.5214
4	0.0003	0.0003	1.0000		0.5214	0.5214
5	<.0001	<.0001	0.5214	0.5214		1.0000
6	<.0001	<.0001	0.5214	0.5214	1.0000	

Statistic Analysis of Free Whey in Yogurts with Sodium Caseinate

F3

The SAS System
The GLM Procedure

Class Level Information

Class	Levels	Values
control	2	control factori
additive	3	Nac TMP none
active	2	active inactive
enzyme	5	0 10 50 100 200

Number of observations 75

NOTE: Due to missing values, only 72 observations can be used in this analysis.

Dependent Variable: Free whey

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	27610.65712	1624.15630	215.01	<.0001
Error	54	407.90353	7.55377		
Corrected Total	71	28018.56064			

R-Square	Coeff Var	Root MSE	force Mean
0.985442	4.162117	2.748412	66.03400

Source	DF	Type I SS	Mean Square	F Value	Pr > F
control	1	18422.98084	18422.98084	2438.91	<.0001
enzyme(control)	4	2167.79704	541.94926	71.75	<.0001
active(control)	2	659.45702	329.72851	43.65	<.0001
additive(control)	1	5213.98104	5213.98104	690.25	<.0001
activ*enzyme(contro)	4	159.26654	39.81663	5.27	0.0012
addit*enzyme(contro)	4	710.76983	177.69246	23.52	<.0001
addit*active(contro)	1	276.40481	276.40481	36.59	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
control	1	14497.36904	14497.36904	1919.22	<.0001
enzyme(control)	4	2167.79704	541.94926	71.75	<.0001
active(control)	2	659.45702	329.72851	43.65	<.0001
additive(control)	1	5213.98104	5213.98104	690.25	<.0001
activ*enzyme(contro)	4	159.26654	39.81663	5.27	0.0012
addit*enzyme(contro)	4	710.76983	177.69246	23.52	<.0001
addit*active(contro)	1	276.40481	276.40481	36.59	<.0001

Level of active	Level of enzyme	Level of control	N	Mean	Std Dev
active	0	control	9	30.3836667	1.2584595
inactive	0	control	3	29.9116667	1.0495010
active	0	factori	6	69.1466667	7.2150140
active	10	factori	6	71.2883333	4.9656154
active	50	factori	6	76.4250000	10.8284159
active	100	factori	6	85.7500000	14.9316362
active	200	factori	6	79.8983333	3.0228623
inactive	0	factori	6	63.2050000	9.8338899
inactive	10	factori	6	62.1033333	12.2219960
inactive	50	factori	6	75.7233333	19.4109801
inactive	100	factori	6	78.2633333	14.5187350
inactive	200	factori	6	70.0733333	7.6886349

Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

active	enzyme	control	LSMEAN force LSMEAN	Number
active	0	control	30.3836667	1
inactive	0	control	29.9116667	2
active	0	factori	69.1466667	3
active	10	factori	71.2883333	4
active	50	factori	76.4250000	5
active	100	factori	85.7500000	6
active	200	factori	79.8983333	7
inactive	0	factori	63.2050000	8
inactive	10	factori	62.1033333	9
inactive	50	factori	75.7233333	10
inactive	100	factori	78.2633333	11
inactive	200	factori	70.0733333	12

Least Squares Means for effect activ*enzyme(contro)
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: force

i/j	1	2	3	4	5	6
1		1.0000	<.0001	<.0001	<.0001	<.0001
2	1.0000		<.0001	<.0001	<.0001	<.0001

3	<.0001	<.0001		0.9680	0.0015	<.0001
4	<.0001	<.0001	0.9680		0.0785	<.0001
5	<.0001	<.0001	0.0015	0.0785		<.0001
6	<.0001	<.0001	<.0001	<.0001	<.0001	
7	<.0001	<.0001	<.0001	<.0001	0.5644	0.0238
8	<.0001	<.0001	0.0203	0.0003	<.0001	<.0001
9	<.0001	<.0001	0.0024	<.0001	<.0001	<.0001
10	<.0001	<.0001	0.0062	0.2113	1.0000	<.0001
11	<.0001	<.0001	<.0001	0.0028	0.9900	0.0010
12	<.0001	<.0001	1.0000	0.9997	0.0095	<.0001

Least Squares Means for effect activ*enzyme(contro)
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: force

i/j	7	8	9	10	11	12
1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	0.0203	0.0024	0.0062	<.0001	1.0000
4	<.0001	0.0003	<.0001	0.2113	0.0028	0.9997
5	0.5644	<.0001	<.0001	1.0000	0.9900	0.0095

Statistical Analysis of Gel Strength in yogurts with Sodium Caseinate

F4

The GLM Procedure

Class Level Information

Class	Levels	Values
control	2	control factori
additive	3	Nac TMP none
active	2	active inactive
enzyme	5	0 10 50 100 200

Number of observations 75

NOTE: Due to missing values, only 72 observations can be used in this analysis.

The SAS System
The GLM Procedure

Dependent Variable: Gel Strength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	27610.65712	1624.15630	215.01	<.0001
Error	54	407.90353	7.55377		
Corrected Total	71	28018.56064			

R-Square	Coeff Var	Root MSE	force Mean
0.985442	4.162117	2.748412	66.03400

Source	DF	Type I SS	Mean Square	F Value	Pr > F
control	1	18422.98084	18422.98084	2438.91	<.0001
enzyme(control)	4	2167.79704	541.94926	71.75	<.0001
active(control)	2	659.45702	329.72851	43.65	<.0001
additive(control)	1	5213.98104	5213.98104	690.25	<.0001
activ*enzyme(contro)	4	159.26654	39.81663	5.27	0.0012
addit*enzyme(contro)	4	710.76983	177.69246	23.52	<.0001
addit*active(contro)	1	276.40481	276.40481	36.59	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
control	1	14497.36904	14497.36904	1919.22	<.0001

enzyme(control)	4	2167.79704	541.94926	71.75	<.0001
active(control)	2	659.45702	329.72851	43.65	<.0001
additive(control)	1	5213.98104	5213.98104	690.25	<.0001
activ*enzyme(contro)	4	159.26654	39.81663	5.27	0.0012
addit*enzyme(contro)	4	710.76983	177.69246	23.52	<.0001
addit*active(contro)	1	276.40481	276.40481	36.59	<.0001

Level of active	Level of enzyme	Level of control	N	Mean	Std Dev
active	0	control	9	30.3836667	1.2584595
inactive	0	control	3	29.9116667	1.0495010
active	0	factori	6	69.1466667	7.2150140
active	10	factori	6	71.2883333	4.9656154
active	50	factori	6	76.4250000	10.8284159
active	100	factori	6	85.7500000	14.9316362
active	200	factori	6	79.8983333	3.0228623
inactive	0	factori	6	63.2050000	9.8338899
inactive	10	factori	6	62.1033333	12.2219960
inactive	50	factori	6	75.7233333	19.4109801
inactive	100	factori	6	78.2633333	14.5187350
inactive	200	factori	6	70.0733333	7.6886349

Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

active	enzyme	control	LSMEAN force LSMEAN	Number
active	0	control	30.3836667	1
inactive	0	control	29.9116667	2
active	0	factori	69.1466667	3
active	10	factori	71.2883333	4
active	50	factori	76.4250000	5
active	100	factori	85.7500000	6
active	200	factori	79.8983333	7
inactive	0	factori	63.2050000	8
inactive	10	factori	62.1033333	9
inactive	50	factori	75.7233333	10
inactive	100	factori	78.2633333	11
inactive	200	factori	70.0733333	12

Least Squares Means for effect activ*enzyme(contro)
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: force

i/j	1	2	3	4	5	6	
1		1.0000	<.0001	<.0001	<.0001	<.0001	
2	1.0000		<.0001	<.0001	<.0001	<.0001	
3	<.0001	<.0001		0.9680	0.0015	<.0001	
4	<.0001	<.0001	0.9680		0.0785	<.0001	
5	<.0001	<.0001	0.0015	0.0785		<.0001	
6	<.0001	<.0001	<.0001	<.0001	<.0001		
7	<.0001	<.0001	<.0001	<.0001	0.5644	0.0238	

8	<.0001	<.0001	0.0203	0.0003	<.0001	<.0001
9	<.0001	<.0001	0.0024	<.0001	<.0001	<.0001
10	<.0001	<.0001	0.0062	0.2113	1.0000	<.0001
11	<.0001	<.0001	<.0001	0.0028	0.9900	0.0010
12	<.0001	<.0001	1.0000	0.9997	0.0095	<.0001

Least Squares Means for effect activ*enzyme(contro)
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: force

i/j	7	8	9	10	11	12
1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	0.0203	0.0024	0.0062	<.0001	1.0000
4	<.0001	0.0003	<.0001	0.2113	0.0028	0.9997
5	0.5644	<.0001	<.0001	1.0000	0.9900	0.0095

Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Least Squares Means for effect activ*enzyme(contro)
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: force

i/j	7	8	9	10	11	12
6	0.0238	<.0001	<.0001	<.0001	0.0010	<.0001
7		<.0001	<.0001	0.2888	0.9962	<.0001
8	<.0001		0.9999	<.0001	<.0001	0.0035
9	<.0001	0.9999		<.0001	<.0001	0.0003
10	0.2888	<.0001	<.0001		0.9013	0.0339
11	0.9962	<.0001	<.0001	0.9013		0.0002
12	<.0001	0.0035	0.0003	0.0339	0.0002	

Statistical Analysis of Free Whey in yogurts with Sodium Caseinate and milk Protein Concentrate (Week 1)

F5

Class Level Information

Class	Levels	Values
control	2	control factori
additive	3	Nac TMP none
active	2	active inactive
temp	2	37 55
enzyme	3	0 50 100

Number of observations 103

NOTE: Due to missing values, only 96 observations can be used in this analysis.

The GLM Procedure

Dependent Variable: whey whey

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	20	1806.195534	90.309777	27.88	<.0001
Error	75	242.963890	3.239519		
Corrected Total	95	2049.159424			

R-Square	Coeff Var	Root MSE	whey Mean
0.881432	57.63222	1.799866	3.123021

Source	DF	Type I SS	Mean Square	F Value	Pr > F
control	1	276.0270920	276.0270920	85.21	<.0001
enzyme(control)	2	298.5484778	149.2742389	46.08	<.0001
active(control)	2	1.1425681	0.5712840	0.18	0.8387
temp(control)	2	802.3018792	401.1509396	123.83	<.0001
additive	1	111.1789014	111.1789014	34.32	<.0001
activ*enzyme	2	4.1595111	2.0797556	0.64	0.5291
temp*enzyme	2	162.8306333	81.4153167	25.13	<.0001
addit*enzyme	2	75.2133444	37.6066722	11.61	<.0001
active*temp	2	1.0927347	0.5463674	0.17	0.8451
addit*active	1	0.5253125	0.5253125	0.16	0.6883
additi*temp	1	68.8942347	68.8942347	21.27	<.0001
acti*temp*enzy	2	4.2808444	2.1404222	0.66	0.5195

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

temp	enzyme	control	LSMEAN whey	LSMEAN	Number
37	0	control	1.1466667		1
55	0	control	10.9733333		2
37	0	factori	1.1450000		3
37	50	factori	0.0083333		4
37	100	factori	0.0000000		5
55	0	factori	8.8558333		6
55	50	factori	2.0575000		7
55	100	factori	0.7975000		8

Statistical Analysis of Free Whey in yogurts with Sodium Caseinate and milk Protein Concentrate (Week 2)

F6

Class Level Information

Class	Levels	Values
Control	2	control factori
Additive	3	NacTMP none
Active	2	active inactive
Temp	2	37 55
Enzyme	3	0 50 100

Number of observations 103

NOTE: Due to missing values, only 96 observations can be used in this analysis.

The GLM Procedure

Dependent Variable: sqwhey

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	20	158.8454896	7.9422745	30.17	<.0001
Error	75	19.7437055	0.2632494		
Corrected Total	95	178.5891951			

R-Square	Coeff Var	Root MSE	sqwhey Mean
0.889446	33.58805	0.513078	1.527562

Source	DF	Type I SS	Mean Square	F Value	Pr > F
control	1	67.90516696	67.90516696	257.95	<.0001
enzyme	2	3.21803319	1.60901659	6.11	0.0035
active	2	10.70996603	5.35498302	20.34	<.0001
temp(control)	2	36.85717902	18.42858951	70.00	<.0001
additive	1	4.17604873	4.17604873	15.86	0.0002
activ*enzyme	2	4.34618594	2.17309297	8.25	0.0006
temp*enzyme	2	4.02545784	2.01272892	7.65	0.0010
addit*enzyme	2	6.56550143	3.28275071	12.47	<.0001
active*temp	2	9.09958046	4.54979023	17.28	<.0001
addit*active	1	0.40849596	0.40849596	1.55	0.2168
additi*temp	1	8.56435548	8.56435548	32.53	<.0001
acti*temp*enzy	2	2.96951852	1.48475926	5.64	0.0052

The GLM Procedure

Least Squares Means
Adjustment for Multiple Comparisons: Tukey

active	enzyme	sqwhey control	LSMEAN LSMEAN	Number
active	0	control	2.98428321	1
inactive	0	control	2.98428321	2
active	0	factori	0.65589916	3
active	50	factori	1.18001236	4
active	100	factori	0.13301175	5
inactive	0	factori	1.19935510	6
inactive	50	factori	1.49679603	7
inactive	100	factori	1.58685576	8

Least Squares Means
Adjustment for Multiple Comparisons: Tukey

sqwhey	LSMEAN temp	enzyme	control	LSMEAN	Number
	37	0	control	2.98603086	1
	55	0	control	2.98253556	2
	37	0	factori	0.32866964	3
	37	50	factori	0.29322734	4
	37	100	factori	0.35764358	5
	55	0	factori	1.52658462	6
	55	50	factori	2.38358105	7
	55	100	factori	1.36222393	8

Statistic Analysis of Gel Strength in Yogurts with Sodium Caseinate and Milk Protein Concentrate (NacTMP)

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Class Level Information

Class	Levels	Values
Control	2	control factori
Additive	3	NacTMP none
Active	2	active inactive
Temp	2	37 55
Enzyme	3	0 50 100

Number of observations 103

NOTE: Due to missing values, only 96 observations can be used in this analysis.

The SAS System

The GLM Procedure

Dependent Variable: sqforce

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	20	77.39889564	3.86994478	72.59	<.0001
Error	75	3.99828357	0.05331045		
Corrected Total	95	81.39717921			

R-Square	Coeff Var	Root MSE	sqforce Mean
0.950879	3.547123	0.230891	6.509234

Source	DF	Type I SS	Mean Square	F Value	Pr > F
control	1	38.91230203	38.91230203	729.92	<.0001
enzyme(control)	2	5.75117923	2.87558962	53.94	<.0001
active(control)	2	1.22982445	0.61491223	11.53	<.0001
temp(control)	2	24.00942798	12.00471399	225.19	<.0001
additive(control)	1	0.24612013	0.24612013	4.62	0.0349
activ*enzyme	2	0.48752158	0.24376079	4.57	0.0134
temp*enzyme	2	4.79744313	2.39872157	45.00	<.0001
addit*enzyme	2	0.51839142	0.25919571	4.86	0.0103
active*temp	2	0.14874820	0.07437410	1.40	0.2542
addit*active	1	0.16065831	0.16065831	3.01	0.0867
additi*temp	1	0.94618467	0.94618467	17.75	<.0001
acti*temp*enzyme	2	0.19109451	0.09554725	1.79	0.1736

Least Squares Means
Adjustment for Multiple Comparisons: Tukey

sqforce active	LSMEAN enzyme	control	LSMEAN	Number
active	0	control	5.40650630	1
inactive	0	control	5.40650630	2
active	0	factori	6.65446541	3
active	50	factori	7.09088359	4
active	100	factori	7.27716432	5
inactive	0	factori	6.43449627	6
inactive	50	factori	6.61044290	7
inactive	100	factori	7.19341076	8

Least Squares Means for effect activ*enzyme(contro)
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: sqforce

i/j	1	2	3	4	5	6	7	8
1	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
2	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
3	<.0001	<.0001		0.0004	<.0001	0.2897	0.9998	<.0001
4	<.0001	<.0001	0.0004		0.5045	<.0001	<.0001	0.9575
5	<.0001	<.0001	<.0001	0.5045		<.0001	<.0001	0.9863

6	<.0001	<.0001	0.2897	<.0001	<.0001	0.5776	<.0001
7	<.0001	<.0001	0.9998	<.0001	<.0001	0.5776	<.0001
8	<.0001	<.0001	<.0001	0.9575	0.9863	<.0001	<.0001

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

sqforce temp	LSMEAN enzyme	control	LSMEAN	Number
37	0	control	5.32896679	1
55	0	control	5.48404581	2
37	0	factori	6.84058868	3
37	50	factori	7.36295651	4
37	100	factori	8.15406373	5
55	0	factori	6.24837301	6
55	50	factori	6.33836999	7
55	100	factori	6.31651135	8

Statistical Analysis of ANOVA in Petit Suisse treated with Transglutaminase

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The GLM Procedure
Class Level Information

Class	Levels	Values
Day	2	1 15
Active	2	active inactive
Temperature	1	37
Enzyme	3	0 50 100

Number of Observations 41

Note: Due to missing values, only 36 observations can be used in this analysis

The GLM Procedure

Dependent Variable: sqforce

Source	DF	Squares	Mean square	F Value	Pr>F
Model	7	644.8626378	92.1232340	199.45	<.0001
Error	28	12.9329334	0.4618905		
Corrected Total	35	657.7955713			

R-Square	Coeff Var	Root MSE	sqforce Mean
0.980339	7.64286	0.679625	8.892285

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Active	1	14.7860799	14.7860799	32.01	<.0001
Enzyme	2	624.7975114	312.3987557	676.35	<.0001
Temp	0	0.000000000	.	.	.
Day*active	2	4.8776160	2.4388080	5.28	0.0113
Active*enzyme2		0.4014306	0.2007153	0.43	0.6518

The GLM Procedure

Level of Day	Level of active	……sqforce…… N	Mean	Std Dev
Active	0	6	6.6473467	0.62891790
Active	50	6	6.3858164	0.49317137
Active	100	6	15.5663257	1.45072572
Inactive	0	6	5.4308604	0.10250105
Inactive	50	6	5.3238345	0.11110368
Inactive	100	6	13.9995268	0.89213147

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

Squforce Active	enzyme	LSMEAN LSMEAN	Number
Active	0	6.6473467	1
Active	50	6.3858164	2
Active	100	15.5663257	3
Inactive	0	5.4308604	4
Inactive	50	5.3238345	5
Inactive	100	13.9995268	6