SIK-Report
1993 No. 592

FUNCTIONAL PROPERTIES OF WHEY PROTEINS

Scientific background and patent applications in low-fat products

Pernilla Walkenström

This review is part of a PhD thesis at the Department of Food Science, Chalmers University of Technology, Sweden

Supervisor: Anne-Marie Hermansson

Göteborg 1993

ISBN 91-7290-153-5

A-nr 840
CONTENTS

1. INTRODUCTION 5

2. DENATURATION 8
   2.1 Theory of denaturation 9
   2.2 Association and dissociation reactions prior to denaturation 12
       2.2.1 $N_2 \rightleftharpoons 2N$ 12
       2.2.2 $4N_2 \rightleftharpoons N_8$ 13
       2.2.3 $N \rightleftharpoons R$ 13
       2.2.4 Aggregation prior to denaturation 13
   2.3 Reversible denaturation 15
   2.4 Forces involved in denaturation 16
   2.5 Denaturation behaviour 18
       2.5.1 Differential scanning calorimetry measurements 19
       2.5.2 Heat stability of whey proteins 21
   2.6 Environmental effects 23
       2.6.1 Effect of pH 23
       2.6.2 Effect of salts 24
       2.6.3 Effect of sugars and polyhydric alcohols 25

3. AGGREGATION 26
   3.1 Characterisation of aggregates 26
       3.1.1 Temperature dependence 28
   3.2 Environmental effects 30
       3.2.1 Effect of pH 30
       3.2.2 Effect of salts 31
       3.2.2.1 $Ca^{2+}$-ions 31
       3.2.2.2 $Na^+$-ions 34

4. EMULSIONS 35
   4.1 Adsorption at interface 37
       4.1.1 Theory of adsorption 37
       4.1.2 Interfacial behaviour 39
       4.1.3 Adsorption at metal faces 43
       4.1.4 Kinetics of adsorption 44
4.1.5 Selectivity in adsorption 46

4.2 Surface forces 49
  4.2.1 Interactions due to adsorption of polymers 50
    4.2.1.1 Irreversibly adsorbed polymers 50
    4.2.1.2 Reversibly adsorbed polymers 51
    4.2.1.3 Non-adsorbing polymers 51

4.3 Emulsifying properties 53
  4.3.1 Effect of hydrophobicity 53
  4.3.2 Effect of solubility 55
  4.3.3 Effect of flexibility 55

4.4 Characterization of emulsifying properties 58
  4.4.1 Emulsifying capacity 58
  4.4.2 Emulsifying activity 59
  4.4.3 Emulsion stability 60
    4.4.3.1 Creaming 61
    4.4.3.2 Flocculation 62
    4.4.3.3 Coalescence 63
    4.4.3.4 Phase inversion/separation 64

4.5 Factors affecting emulsion stability 65
  4.5.1 Droplet size 65
  4.5.2 The nature of the interfacial film 67
  4.5.3 Liquid crystals 69
  4.5.4 The continuous medium 69
  4.5.5 Miscellaneous factors 70

4.6 Classifying emulsifiers 71
  4.6.1 HLB-system 71
  4.6.2 PIT-phase inversion temperature 72

5. GELS 73

5.1 Gelation 75
  5.1.1 Gelation mechanisms 75
  5.1.2 Kinetics of gelation 77
  5.1.3 Gel point 78

5.2 Forces in a gel 82
  5.2.1 Theory 82
  5.2.2 Covalent forces 84
  5.2.3 Hydrophobic interactions 85
  5.2.4 Electrostatic and ionic forces 85
  5.2.5 Reversibility of whey protein gels 86
5.3 Microstructure of whey protein gels

5.3.1 Fine-stranded gels

5.3.2 Particulate gels

5.3.3 Fractal structure

5.4 Rheology of whey protein gels

5.4.1 Basic rheological definitions

5.4.1.1 Non-destructive tests

5.4.1.2 Destructive tests

5.4.1.3 Viscosity

5.5 Viscoelastic properties of whey protein gels

5.5.1 Effect of heating condition

5.5.2 Effect of protein concentration

5.5.3 Effect of pH

5.5.4 Effect of salt

5.5.5 Effect of different environmental factors

6. APPLICATIONS IN THE PATENT LITERATURE

6.1 Emulsions

6.2 Particles

7. CONCLUSIONS

8. REFERENCES
1. INTRODUCTION

Whey was traditionally regarded as a waste product and was generally disposed of as animal food. However, during the past few decades, whey has attracted considerable attention as a potential food ingredient. This is primarily because of its excellent nutritional value and promising functional properties in a range of food products. The functional properties receiving most attention are those of emulsifying and gelling.

Whey includes a characteristic group of different globular proteins. These have been grouped together because they remain in the whey during cheese making and, unlike the caseins, they remain in the solution at pH 4.6. The various whey proteins differ both in structure and in properties as a result of differences in the amino acid sequence. In table 1 the whey proteins and some of their properties are depicted. In this review, the different whey proteins will be abbreviated as follows: β-lg for β-lactoglobulin, α-la for α-lactalbumin and BSA for bovine serum albumin. In the table, abbreviations for the immunoglobulins are already set (Ig), and the proteose peptones will be shortened to PP throughout this work. β-lg is the dominant protein in whey. It comprises up to 50% of the total protein content and its behaviour governs the whole whey. This is the main reason why more studies are being performed on β-lg than on the other whey proteins. Six genetic variants have been discovered for β-lg, the A and B variant being the most abundant, and two for α-la, outlined in table 1 (Mulvihill and Donovan, 1987). The individual proteins in whey and their behaviour have been investigated and reviewed by (Mulvihill and Donovan, 1987; McKenzie, 1971; Gordon, 1971).

From an industrial point of view, there are two main types of whey: sweet and acid whey. These are by-products of cheese or rennet casein and acid casein, respectively. Sweet whey has a minimum pH of 5.6 and acid whey a maximum pH of 5.1 (IDF, 1978). The acidification is obtained either by direct addition of mineral acid or by in situ production of acid by added starter bacteria. The two types differ from each other in their respective composition, for example in Ca²⁺-content. Acid whey may contain 2.5-3 times more calcium than sweet whey and this will affect its behaviour to a great deal, especially with regard to aggregation phenomena, see section 3.2.2.1. De Wit et al made an evaluation of the functional properties of whey protein concentrates and whey protein isolates and explained the objectives of a comparative, functional characterization of commercially available whey protein concentrates (De Wit et al, 1983).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight (Da)</th>
<th>Sedimentation constant ($S_{20w}$)</th>
<th>Isoionic point</th>
<th>Isoelectric point</th>
<th>Genetic variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lg</td>
<td>18,205-18,363</td>
<td>1.9-2.7</td>
<td>5.35-5.41</td>
<td>5.13</td>
<td>A,B,C, D, ADR, BDR</td>
</tr>
<tr>
<td>α-la</td>
<td>14,147-14,175</td>
<td>1.87-1.96</td>
<td>—</td>
<td>4.2-4.5</td>
<td>A,B</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>66,267</td>
<td>4.0-5.01</td>
<td>5.13-5.15</td>
<td>4.7-4.9</td>
<td>A</td>
</tr>
<tr>
<td>Ig G₁</td>
<td>153,000-163,000</td>
<td>6.3-7.0</td>
<td>—</td>
<td>5.5-6.8</td>
<td></td>
</tr>
<tr>
<td>Ig G₂</td>
<td>146,000-154,000</td>
<td>6.5-7.1</td>
<td>—</td>
<td>7.5-8.3</td>
<td></td>
</tr>
<tr>
<td>Ig A</td>
<td>385,000-417,000</td>
<td>10.8-11.0</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Ig M</td>
<td>960,000-1,000,000</td>
<td>18.2-19.8</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Ig E</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PP Component 3</td>
<td>40,800</td>
<td>1.6</td>
<td>3.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PP Component 5</td>
<td>14,300</td>
<td>1.22</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PP Component 8-fast</td>
<td>4,100</td>
<td>0.78</td>
<td>3.3</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PP Component 8-slow</td>
<td>9,900</td>
<td>1.35</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Proteins of whey and some of their properties (Mulvihill and Donovan, 1987, adapted from Whitney et al, 1976 and Eigel et al, 1984).

Whey is heat-sensitive and often exposed to heat for various reasons, usually during the manufacturing of whey protein concentrates during processing of food products. Heat treatments will denature the whey protein. During denaturation the protein will unfold. This will alter the conformation of the protein and secondary reactions, such as aggregation, will take place. Aggregation is a complex reaction which affects the gelling and emulsifying ability of the proteins. It is therefore important to be able to control and guide the denaturation and aggregation mechanisms in order to obtain the desired product properties. A knowledge of the stability of the functional properties of whey proteins in different environments and under special conditions, for example in food products, is also important in order to preserve the desired effects.

The second chapter in this review deals with denaturation of whey proteins, specially heat denaturation. Forces involved and environmental factors are considered as well.
as association and dissociation reactions of β-lg prior to denaturation. Denaturation is a well researched area and a lot of scientific publications exist. Aggregation is the topic of the third chapter. This is an area where there are relatively few publications and more work is needed.

Emulsions are discussed in chapter 4. The adsorption at interfaces, forces involved and emulsifying properties are briefly touched upon as well as the stability of emulsions. The chapter deals with both emulsions, i.e. o/w- and w/o-emulsions, and foams in a mixed fashion. In chapter 5 whey protein gels are addressed. Gelation mechanisms, forces involved, microstructure and viscoelastic properties of the gels are discussed. Section 5.4.1 also covers basic rheological definitions.

The last chapter deals with patents. It illustrates the use of whey proteins as a substitute for fat in different low-fat products, mostly spreads. In the patents, the protein is used in emulsions or as specially sized particles, which is achieved with the help of different parameters, for example pH, temperature, shearing forces, salt, etc. In emulsions, the gelling and emulsifying properties of whey are used.

The chapter on patents clearly brings out the need, of the food industry, for improved processes regarding production of suitably sized and shaped particles. With reference to specially sized particles, little work has been published. Denaturation and aggregation are required in this case, but the mechanisms regarding aggregation are not clear, as little research has been done in this area. As a result, the aggregation is not easily controlled and the specially sized particles, which give the desired organoleptic character, are hard to obtain.

With present scientific and technological developments, it may be possible to determine the kinetics and mechanisms of whey protein aggregation and, by this means, control and guide the aggregation in order to increase whey protein utilization by developing new products and also remove the present limitations of the whey protein materials as food additives.
2. DENATURATION

This chapter will throw some light on heat denaturation of whey proteins. Possible association and dissociation reactions, which may take place before denaturation, will also be discussed. The denaturation is usually achieved by heat treatment and by varying some other factors, such as pH, salt concentration etc.

As mentioned before, the denaturation process usually alters the functional properties of the protein. A major consequence is reduction in protein solubility. If we go to another part of this work, "Application in the patent literature", we find that this is desired. We want reduced solubility, and we want to control and guide it to either specially sized particles or to obtain some gelling, emulsifying and/or foaming properties. Figure 1 show the main forces involved in determining these functional properties of food proteins. It also points out that other molecules present and extrinsic factors may affect protein structure and conformation.

![Diagram](image)

Fig.1 Factors and forces involved in determining the functional properties of food proteins (Kinsella and Whitehead, 1989).
2.1 Theory of denaturation

Denaturation is a phenomenon that is intimately linked to the conformational state of a protein and, therefore, also with its stability and functionality. It affects the preparation, processing, nutritional value, quality, safety, aggregation and gelling ability, foaming and emulsifying properties of food proteins. One basic requirement is a knowledge of the denaturation behaviour of individual proteins in different mediums to be able to set operating conditions to produce the desired effect in products.

Many definitions of denaturation have been made. One is "away from the native state" (Mulvihill and Donovan, 1987). This definition is broad and open to many interpretations. More precisely defined definitions have also been made, such as:

"Denaturation involves conformational changes from the native structure without alteration of the amino acid sequence" (Tanford, 1968)

"Any major alteration in the original native structure without hydrolysis of primary covalent bonds, i.e. changes are restricted to those occurring in secondary or higher structure" (Mulvihill and Donovan, 1987)

These definitions are not absolute. For example "any major alteration" is not very specific. Modern theories suggest that the conformation of a protein is determined by its amino acid sequence and environment, i.e. the unique three-dimensional structure of a protein is the result of various attractive and repulsive interactions of the protein chain within itself and with the surrounding solvent (Mulvihill and Donovan, 1987). Proteins also fold to gain their most energetically favourable conformation, i.e. the conformation is thermodynamically controlled (Brandt, 1967).

The protein can also go through a small change and adapt an energetically equivalent state, called "quasi-native" state. This small change can easily be overcome and does not refer to a denaturation as such. The various alterations the protein adopts depend on the forces maintaining and stabilizing the native conformation. These forces are hydrogen bonding and hydrophobic and electrostatic interactions. When a protein is denatured, these forces lead to a labile, disordered state. The transition from the native ordered state to a denatured disordered state involves marginal energy differences and may be overcome by small alterations in the surrounding medium. This is possible because the free energy change from the stabilizing interactions just counterbalances the large conformational entropy of the disordered random state.
Denaturation is involved in most structure-forming processes. When the forces that stabilize the native state are ruptured, the native protein conformation collapses and the result is a new configuration due to denaturation. It is important to control the denaturation process in order to obtain structures with the desired textural properties and for technical and commercial success in whey processing and utilization.

The denaturation process is reversible and co-operative. The co-operativity arises because the transitions are steep and occur within a narrow range of temperature or concentration of denaturants (Tanford, 1968). The $Q_{10}$-value is also high for protein denaturation (Donovan and Mulvihill, 1987). The $Q_{10}$-value serves as a measure of the relative shelf-life of a product, in this case denaturation, between any two temperatures 10°C apart. It is defined as:

$$Q_{10} = \frac{\theta_{s,T}}{\theta_{s,T-10}}$$  \hspace{1cm} (1)

where $\theta_s$ is the shelf-life at T and at T+10°C, respectively (Labuza, 1985).

The degree of denaturation depends on both the protein and the conditions involved. Denaturation can be restricted to a segment or to the complete molecule. In the case of denaturing the whole molecule, it can be fitted to a two-state hypothesis (Brandts 1967): native(N)$\rightarrow$denatured(D). This two-state denaturation is graphically illustrated in figure 2, where the native and denatured states are thermodynamic states which result from a distribution of microscopic states. These microscopic states represent different degrees of structural order in the polypeptide chain and differences in the structure of water molecules adjacent to the protein. The temperature effect is also illustrated in the figure, and it can be seen that the degree of order of a protein molecule decreases as the temperature increases (Mulvihill and Donovan, 1987).

As mentioned above, the denaturation process is a reversible process, but because of factors and subsequent reactions after denaturation, the whole process may become operationally irreversible. These irreversible steps usually take place above the denaturation temperature ($T_D$) (De Wit, 1989a). In the denaturation step, the protein unfolds itself and chain-solvent interactions are favoured (Hermansson, 1979). New binding sites, which had previously been buried in the native protein or interacted within the native molecule are now exposed. These sites are now free to interact with sites on other protein molecules, leading to intermolecular associations and aggregation. In most processes, the denaturation is followed by aggregation steps, due to these protein-protein interactions, and subsequent precipitation, coagulation and/or gelation. Thus, those forces involved in stabilizing the native molecule are also
involved in determining the aggregation.

Fig. 2 A graphical representation of a two-state protein denaturation. The relative populations of the numerous microstates of the native (N) and denatured (D) distributions are indicated by the ordinate. The arrows show the average "degree of order" for the two thermodynamic states and indicate the temperature dependence of this factor as the temperature is increased (top to bottom) through the transition period (Mulvihill and Donovan, 1987). (Adapted from Brandts, 1969).
2.2 Association and dissociation reactions prior to denaturation

2.2.1 $2\text{N}^+ \rightarrow \text{N}_2$

Native $\beta$-lg (N) usually exist as a dimer, $\text{N}_2$, at room temperature ($20^\circ\text{C}$) and neutral pH-value, i.e. between pH 5.5 and 7.5. It consists of identical subunits, each with a molecular weight around 18,400 Da. A dynamic equilibrium exists between the dimer, $\text{N}_2$, and monomer, $2\text{N}$ (McKenzie, 1971). The equilibrium is shifted to the monomer when the temperature is raised. This has been studied by many scientists. For example, increased dimer dissociation above 30°C has been demonstrated by optical rotation dispersion measurements (ORD) (McKenzie and Sawyer, 1967; De Wit and Klarenbeek, 1984). This dissociation is also observed at high and low pH-values and is explained by an increasing electrical charge and subsequent electrostatic repulsion (MacKenzie and Sawyer, 1967; Harwalkar, 1980a). Townend et al also studied the dimer-monomer reaction and concluded further that the sulfhydryl groups, like the tyrosine and tryptophan side-chain residues, are not located at the sites of subunit contact and do not affect the monomer-dimer equilibrium (Townend et al, 1969). This dimer behaviour has also been observed by Harwalkar and Kalab. They studied the thermal denaturation of $\beta$-lg at different ionic strengths and at pH 2.5. They suggest that with increasing ionic strength of the medium at this pH-value, the electrostatic repulsion is reduced and the monomers associate to shift the equilibrium between the monomers and dimers in favour of the dimers (Harwalkar and Kalab, 1985b).

Lately, it has been proposed that monomeric $\beta$-lg molecules denaturates only partly and then associates to form the dimer due to exposed hydrophobic areas. This is in agreement with the results obtained on ovalbumin. This globular protein aggregates immediately when partly denatured due to exposure of hydrophobic areas. It was revealed with the help of transmission electron microscopy that linear polymers were formed (Koseki et al, 1989). The $\beta$-lg dimer then denaturates completely and dissociates at the same time to its monomeric form, followed by aggregation (Relkin and Launay, 1990). Mild temperature effects on structure and solubility of whey proteins are reversible and governed mainly by hydrophobic bonding. Hydrophobic bonding is enhanced when temperatures increase up to 60°C and is weakened as temperatures decrease. This implies that proteins with a relatively high amount of hydrophobic residues, i.e. $\beta$-lg, are sensitive to temperature-induced association or dissociation reactions (Payens and Vreeman, 1982).
2.2.2 $4N_2 \rightarrow N_8$

Another dissociation/association reaction takes place for the $\beta$-lg molecule. At low temperatures and between pH 3.7 and 5.1, the dimers self associates to an octamer (Pessen et al., 1989). The equilibrium is rapid and well characterized (Kumosinski and Timasheff, 1966). It has been observed that it occurs to a greater extent with the genetic variant A of $\beta$-lg. With the help of light scattering techniques at 2°C and at pH 4.65, it has been shown that the optimum octamer formation is over 90% complete for $\beta$-lg A versus 31% for $\beta$-lg B (Kumosinski and Timasheff, 1966).

2.2.3 $N \rightleftharpoons R$

A reversible conformational change occurs between pH 6-9 for $\beta$-lg at room temperature. A small change occurs with time and it is immediately established on mixing. This transition has to take place through the monomeric state, and an initial heat effect is to drive the monomer-dimer equilibrium in order to obtain the monomer. The transition is denoted $N \rightleftharpoons R$, R representing the new reversible conformation of the protein. Further studies of the transition have been made with the help of ORD measurements (Tanford et al., 1959; Tanford and Taggart, 1961; Pantaloni, 1963). The $N \rightleftharpoons R$ transition includes a release of a proton from a buried carboxyl group (Tanford et al., 1959; Tanford and Taggart, 1961; Timasheff et al., 1966a,b; Townend et al., 1969), and this is generally considered to be the "ionization linkage" of the transition. Shimada and Cheftel observed an increase in gel transmittance above pH 7, and explained that this was due to the $N \rightleftharpoons R$ transition. They also pointed out that the transition takes place near pH 6.8 in the native state (Shimada and Cheftel, 1988). The transition is also accompanied by titration of histidine residues (McKenzie, 1971), which are titrated in this region, i.e. pH 6-9, increased solvent exposure of tyrosine and tryptophan residues (Townend, et al 1969), as well as increased thiol activity (Dunhill and Green, 1966). The $N \rightleftharpoons R$ conformational change increases with increasing temperature near pH 7.0 (McKenzie, 1971).

2.2.4 Aggregation prior to denaturation

Aggregation of protein molecules prior to denaturation has been observed (Stading and Hermansson, 1990). Stading and Hermansson found an increase in absorbance at 420 nm of $\beta$-lg solutions at 22°C in the pH-range 4.5-6. See figure 3. They explained that this might be some kind of aggregation prior to gelation.
Fig. 3 Absorbance at 420 nm on 0.1% w/w β-lactoglobulin solution at 22°C (Stading and Hermansson, 1990).

They also found that the gelation temperature ($T_g$) was low in this pH-range. It was much lower than $T_d$, indicating that aggregation and gelation took place prior to denaturation (Stading and Hermansson, 1990). This may be described as

$$xN \rightarrow N_x \rightarrow D_x$$

(2)

where $N$ is the native form of the protein, $x$ is the number and, in the case of gels, $N_x$ and $D_x$ are the gel networks of native and denatured molecules, respectively.
2.3 Reversible denaturation

Reversible denaturation is obtained during mild heat treatment and on restoration to the original environmental conditions, the denatured β-lg molecule (D) may revert to its original native configuration (N₂). Around 65°C the transformation of native β-lg monomer to a reversible denatured molecule takes place. It is this transformation that constitutes the primary step in the heat denaturation of β-lg. On the basis of optical rotation measurement ([α]₄₃₅), Dupont (1965a,b) showed that immediate transition took place above 40°C at pH 6.85 with a marked increase in levo-rotation occurring at temperatures above 65°C. The transition was also followed with the help of ultraviolet difference spectral measurements by Pantaloni (1965), with comparable results.

The R→D transition involves exposure of apolar residues to the solvent and breakdown of two different kinds of bonds, hydrogen bonds and hydrophobic bonds. This will result in the loss of tertiary and secondary structures and is defined, according to above-mentioned definitions, as a denaturation. This was verified by optical rotary dispersion (ORD) and UV spectral difference measurements (Dupont, 1965a). The transition was studied with the help of ORD, far-UV ORD and circular dichroism (CD) measurements. It was revealed that an increase in random coil and β-structure was accompanied by unfolding of α-helical structures and that this proceeded to an extent depending on both the time and temperature of heat treatment. Unmasking of the thiol groups also occurs during reversible denaturation (Sawyer et al, 1971). The D-state is governed by high disorder and entropy, and the molecules are prone to further interactions and this is the main reason for subsequent reactions and irreversible steps.

The steps in conformational changes outlined so far are reversible and occur either at room temperature or during mild heat treatments. They may be summarized as:

\[ N₈ = 4N₂ = 8N = 8R = 8D \]  \hspace{1cm} (3)

N represents the native molecule, N₂ the native dimer and R the reversible conformational change that takes place at room temperature, discussed above. D symbolizes the reversible denatured state, previously discussed.
2.4 Forces involved in denaturation

During heating the stabilizing forces and bonds, i.e. hydrogen, hydrophobic and electrostatic bonds and forces, in the native protein are ruptured or weakened (Hardy, 1899). This results in the destruction of secondary structures and denaturation. Increasing temperatures also have a disorganizing effect on water structure by causing deformation of the hydrogen bonds connecting water molecules (von Hippel and Schleich, 1969). An aqueous environment is essential for maintenance of native structure, and heat-induced alterations of the solvent character contribute to protein instability. An increase in the frequency of molecular collision also results from increased kinetic energy at high temperatures. This increase in collisions enhances thiol-disulfide interactions, which promotes denaturation (Li Chan, 1983).

β-Lg contains two disulfide bonds and one free thiol group per monomer. During conformational change the thiol group may be capable of interacting and forming a new disulfide bond, either intermolecularly or intramolecularly, which may affect the solubility and functionality of the protein (Dunhill and Green, 1966). It has been proved that increased thiol-disulfide reactions are achieved under heating conditions (Li-Chan, 1983 and Shimada and Chettel, 1989). If the activity of the thiol groups is sufficiently high, the formation of intramolecular disulfide bonds will, at low protein concentration, prevail over intermolecular disulfide interactions (Creighton, 1978).

![Graph showing the effect of heating temperature on reactive thiol group formation](image)

**Fig. 4** Effect of heating temperature [60°C (△), 70°C (□), 80°C (■), 90°C (○)] and time (0, 1, 3, 5, 10 min) on reactive thiol group formation in a rennet casein whey heated at pH 6.7 (Donovan and Mulvihill, 1987).
It is also found, in agreement, that intermolecular predominates over intramolecular disulfide bonds at a relatively high protein concentration and that this might be the reason for β-lg unfolding during heating (Shimada and ChefTel, 1989).

Denaturation may sometimes be depicted as an increase in the free sulphydryl content. This increase is due to the unfolding of the β-lg molecule and subsequent exposure of buried sulphydryl groups. In figure 4 the denaturation is calculated as the reactive thiol concentration. It is a three-dimensional plot, and time and temperature are also depicted in the figure. On heating at 60-70°C for up to 10 minutes, there was a little alteration in sulphydryl group content. Larger changes occurred at temperatures above 80°C. Rupture of disulfide bonds of α-la, BSA and Ig at high temperatures make a minor contribution to the increase in thiol content. This is a further indication that the denaturation of whey protein follows that of β-lg, since the liberation of sulphydryl groups of whey arise almost from β-lg only.

Interesting results regarding complete exposure of thiol groups have been obtained. When heating β-lg under conditions expected to cause complete denaturation, and thus also to result in complete exposure of thiol groups, the level of reactive thiol groups does not reach the total thiol content. A suggestion for possible reactions of the sulphydryls has been made (Lyster, 1964):

i) they may be lost by volatilization

ii) they may become reburied within protein structures

iii) they may oxidize to disulfides or become involved in sulphydryl disulfide exchange reactions

Suggestions have been made that reaction iii) may have the greatest effect. This is because subsequent cooling after heating could promote further aggregation of the denatured protein and, under these circumstances, one would expect cysteine and/or cystine residues to become involved in aggregate formation (Sawyer, 1968). This discussion clearly shows that the assumption that denaturation is due to an increase in sulphydryl content may be incorrect.
2.5 Denaturation behaviour

The thermal behaviour of whey proteins has been extensively studied, for example, by using ORD, CD (Sawyer et al, 1971), solubility loss (Harwalkar, 1980a,b; Donovan and Mulvihill, 1987) and differential scanning calorimetry (DSC) (Hegg, 1980; De Wit and Swinkels, 1980; Rüegg et al, 1977; Park and Lund, 1984; Relkin and Launay, 1990; Hermansson, 1979; De Wit and Klarenbeek, 1981 and 1984; Paulsson and Dejmek, 1990; Paulsson et al, 1985; Bernal and Jelen, 1985; De Wit et al, 1983). Most investigations have been made by DSC measurements.

As mentioned before, the denaturation step involves a marginal energy difference. This is shown in figure 5 by the sharp transition of the native to the denatured state. It is a three-dimensional plot considering denaturation, temperature and time. The denaturation is measured as solubility loss of nitrogen, N. It is measured after heat treatment at pH 6.7 and subsequent cooling and adjustment to the isoelectric pH-value, i.e. pH 4.5. It is denoted "pH 4.5 insoluble N (as % of total N)". At this pH-value, the denaturation is presumed to be intimately linked with aggregation. It is a usual method to measure denaturation as loss in solubility at the isoelectric zone (Li-Chan, 1983).

Fig.5 Effect of heating temperature [60°C (△), 70°C (□), 80°C (■), 90°C (○)] and time (0,1,3 5 10) on the production of pH 4.5-insoluble nitrogen (N) in a rennet casein whey heated at pH 6.7 (Donovan and Mulvihill, 1987).
The curves in the figure have a sigmoidal shape, indicating the sharp transition occurring under these environmental conditions around 90°C. It also indicates that denaturation is a co-operative process, and the steepness of the curves clearly illustrates the high $Q_{10}$-value for protein denaturation.

2.5.1 Differential scanning calorimetry measurements

DSC-measurement of heat denaturation of whey protein and β-lg is a widespread and simple technique. The conformational changes associated with denaturation are endothermic, and this heat effect is observed in DSC-measurements. It also separates the denaturation phenomenon from the aggregation. This separation is not always obtained with the other methods, and it is possible to obtain wrong values due to the incorrect assumption that denaturation is the same as aggregation. This may also be one of the reasons for the disagreement existing regarding $T_D$-values.

In the DSC-measurements two distinct peaks are noticed (De Wit and Klarenbeek, 1981 and 1984; De Wit, 1981; De Wit and Swinkels, 1980; Paulsson et al, 1985): one near 70°C and one around 130°C at neutral pH-values. In figure 6, DSC-curves for β-lg at various pH-values are shown.

![DSC curves](image)

Fig.6 DSC curves of the heat denaturation of β-lactoglobulin as a function of temperature at pH 3.5 and in the pH-range 6.0<pH>8.0. Protein concentration: $0.16\times10^6$ mol/30µl (De Wit and Klarenbeek, 1981).
In figure 6, these two peaks are observed for pH-values above 6.5. At pH 6.5 and below three peaks are observed, one near 70°C and two around 130°C. The origin of the two peaks around 130°C is not clear, but it has been suggested that they may be related to the native S-S bonds in β-lg (De Wit and Klarenbeek, 1981). The remaining peak at 130°C, above pH 6.5, increase as the pH-value increase. This increase parallels the decrease in the peak at 70°C, and at pH 8 the peaks seem almost of equal size. This relation is also observed in figure 7, where transition temperatures and enthalpy of denaturation are depicted. The figure clearly shows that the transition temperature decreases as the pH-value increases, for both peaks. The figure also shows that the enthalpy of denaturation, for the peak at 80°C, decreases as the pH-value increases.

![Graph](image)

Fig.7 Transition temperatures (a) and calorimetric heats of denaturation near 80°C (b) of β-lactoglobulin solutions at different pH-values. X and O are independently prepared samples (De Wit and Klarenbeek, 1981).

It has been suggested that the decreasing peak size near 80°C and the increasing peak size near 130°C at pH>6.5 could be related to thiol-disulfide chemistry (Watanabe and Klostermeyer, 1976; De Wit and Klarenbeek, 1981). The thiol-disulphide
reactions may restrict complete unfolding near 80°C, and melting of these residual structures might be responsible for the peak at 130°C. This is in agreement with the observed fact that the heat effect arises from identical sources, i.e. denaturation (De Wit and Klarenbeek, 1981). It has also been observed by ORD measurements that, after thermal treatments of β-lg at 90°C, some residual structure remains, even when complications arising from disulfide exchange reactions are eliminated (Ananthanarayanan et al, 1977). Melting of these residual conformations may be due to the endothermal peak observed at 130°C.

2.5.2 Heat stability of whey proteins

The order of heat stability concerning the different whey proteins in neutral pH-range is significantly different. In figure 8, the % denaturation is measured by densitometric scans of electrophoretiograms (Donovan and Mulvihill, 1987). The electrophoretiograms are made of pH 4.5-soluble supernatants from whey samples, heated at 60-90°C for 10 minutes at pH 6.7. One can clearly see in the figure that the order of heat stability is PP>α-la>β-la>BSA>β-lg. Figure 8 also shows that the denaturation of whey protein follows that of β-lg to a great extent.

![Fig.8 Thermodenaturation of total whey protein and of individual whey proteins in rennet casein whey heated at 60-90°C for 10 min at pH 6.7; (▲) Ig, (■) BSA, (α) β-lg, (△) total protein, (●) α-la and (□) PP (Donovan and Mulvihill, 1987).](image-url)
Different results regarding the order of heat stability have been observed with the help of DSC measurements. For example: \( \beta\text{-lg}>\text{Ig G}>\text{BSA}>\alpha\text{-la} \). The heat resistance of whey proteins followed that of \( \beta\text{-lg} \). Consequently, in this case, \( \alpha\text{-la} \) appears to be the least thermostable whey protein (De Wit and Klarenbeek, 1984).

Paulsson et al studied the thermal stability of different whey proteins in the temperature range 25-140°C by DSC. The thermal denaturation temperature \( (T_d) \) and the transition enthalpies \( (-\Delta H_{\text{app}}) \) were determined at different pH-values, 3.0-10.0. The results obtained showed that \( \beta\text{-lg} \) was, except at pH 9.0 and 10.0, the most thermostable protein at all pH-values. At acidic pH-values BSA was the least thermostable. At alkaline pH-values, \( \alpha\text{-la} \) had lower thermal stability than BSA (Paulsson et al, 1985).

The denaturation of whey proteins is affected differently by heating rate, heating time, pH, solvent, ionic strength etc. Thus, lack of uniformity in experimental conditions may be one of the reasons for the different results reached. Also, the different measuring techniques of denaturation may give rise to the different orders obtained. Another explanation for the discrepancies of the results is the high reversibility in denaturation of \( \alpha\text{-la} \). \( \alpha\text{-la} \) is the only heat-denatured whey protein which can be verified by repeating the DSC run after cooling the sample (de Wit and Klarenbeek, 1984). This experiment reveals that the other whey proteins, and also whey protein concentrates, do not show endothermal heat effects of unfolding in a repeated run. These results also demonstrate that \( \alpha\text{-la} \), although it is the whey protein with the lowest denaturation temperature, appears to be (at pH 6.0) most thermostable against protein aggregation because of its high capability of renaturation on cooling (de Wit et al, 1983). The renaturation is of the order of 80-90% (Rüegg et al, 1977). This renaturation effect may, however, be eliminated in a repeated DSC run of whey protein concentrate, due to interaction with \( \beta\text{-lg} \) and BSA (De Wit and Klarenbeek, 1984).
2.6 Environmental effects

Denaturation is a phenomenon that depends on several factors in the environment, for example, heating conditions, pH, salt, protein concentration, protein-modifying agents, total solid concentration, sugar and polyhydric alcohols, thiol-group modification etc. These factors affect the denaturation behaviour in different ways and to different degrees. Some of these factors have been studied more than the others. The main factors and their effects are discussed here.

2.6.1 Effect of pH

The heat denaturation of whey proteins is a pH-dependent process and has been the subject of numerous studies. The pH-dependence is strongly related to those forces stabilizing the tertiary structure of the protein. The strongest influence comes from electrostatic repulsive forces and thiol group activity. It is therefore reasonable to expect variations in the denaturation temperature ($T_D$) of proteins at different pH-values.

In figure 9, DSC-measurements of the $T_D$ of β-lg are depicted at various pH-values. It points out that the protein is most stable against heat denaturation around pH 4, i.e. around its isoelectric pH-value, where the net charge is low. This observation has been further confirmed with the help of DSC-studies (Hermansson, 1979; Bernal and Jelen, 1985).

![denaturation temperature vs pH](image)

Fig.9 The denaturation temperature ($T_D$) of β-lactoglobulin at different pH values determined by DSC. Heating rate: 10°C/min (Hegg, 1980).
De Wit and Klarenbeek made a study of the effects of temperature on denaturation of whey proteins at pH 3.0, 6.0 and 7.5 with the help of DSC. Their results are in agreement with figure 9 in that β-lg shows increased thermostability at pH 3 and decreased stability at pH 7.5 as compared to that at pH 6.0 (De Wit and Klarenbeek, 1984). However, Harwalkar came up with other results. He concluded that the whey proteins denatured to a lesser extent at pH 2.5, and that they were more incompletely denatured than above this pH-value. He did not study this with the help of DSC measurement. He determined the denaturation amount from loss in solubility in 2% trichloro acetic acid (TCA) or at pH 4.5, and thus measured aggregation rather than denaturation (Harwalkar, 1979 and 1980a).

The behaviour at low pH-values has been extensively studied with various techniques, for example, with the help of DSC-measurements (Hermansson, 1979; Bernal and Jelen, 1985; Paulsson, 1990) and as loss in solubility at pH 4.5 or in trichloro acetic acid (Harwalkar, 1979; Harwalkar, 1980a and b; Modler and Emmons, 1976). Disagreements exist regarding the results.

An interesting reaction takes place at pH-values below 3.0. The β-lg dimer reversibly dissociates to its monomeric form, and this is explained to be due to repulsive electrostatic forces developed at this pH (Harwalkar, 1980a).

At pH-values over the isoelectric region, the denaturation temperature decreases, see figure 9 (Hermansson, 1979; Park and Lund, 1984; Paulsson et al , 1985; De Wit and Klarenbeek, 1981). The sulphydryl and disulfide levels of β-lg both decrease above pH 6.9 (Watanabe and Klostermeyer, 1976). This is expected, since it is known that cystine and cysteine residues of proteins in alkaline media are easily converted into degradation products such as H₂S and dehydroalanine (Asquith and Carthew, 1972).

2.6.2 Effect of salts

The denaturation of whey proteins is largely determined by heat and pH, and the effect of salts on heat denaturation is small. The small effect, measured by DSC, is a decrease in T₀ (Varunsatian et al, 1983). The salts and their ions affect the aggregation mechanism of whey proteins to a greater extent and this phenomenon has been the subject of many investigations. (See section 5.2.2.)
2.6.3 Effect of sugars and polyhydric alcohols

It is generally known that sugars and polyhydric alcohols have a stabilizing effect on heat denaturation (De Wit and Klarenbeek, 1981; De Wit, 1981; Bernal and Jelen, 1985; Park and Lund, 1984). The reason for this may be that the substances maintain or increase the hydration of the protein molecule. The water structure is enhanced in the immediate surroundings of the protein (Bull and Breese, 1978). The addition of the substances also results in an unfavourable free-energy change, and this change increases with the increase in protein surface area due to unfolding (Arakawa and Timasheff, 1982).

If a reducing sugar is added, i.e. glucose or lactose, a strong exothermal effect can be observed in the higher temperature interval, 130°C. This is probably a consequence of the reaction between β-lg and the reducing sugar, a Maillard reaction (De Wit and Klarenbeek, 1981).
3. AGGREGATION

3.1 Characterization of aggregates

Little research has been done with regard to the characterization of aggregates and particles. This aspect has been dealt with in some detail in the section on patents in this review, which clearly points out the need for more investigations in the area in order to develop new products and increase whey protein utilization.

Dynamic and static light scattering may be a useful tool for elucidating the initial aggregation mechanism and small aggregates. Dynamic light scattering gives information about translational, rotational and internal motions of macromolecular particles in a solution or suspension. Static light scattering measurements reveal the size and shape of these particles. These two techniques provide complementary information on small aggregates. The relationship between the nature of the heat treatment and the size, flexibility and shape of the protein aggregates formed will reflect the initial molecular details of the mechanism of aggregation. M. Griffin and coworkers in Reading, England, use these techniques to characterize the mechanism of the initial aggregation. Dejmeke's group in Lund, Sweden, is also working in this area. It is a new and developing field, and nothing has been published so far.

In contrast, aggregates and particle sizes constructing gel networks have been studied. For example, Langton and Hermansson (1992) studied the effects of pH on the gel network of β-lg with the help of different microscopic techniques. This will be discussed in section 5.3.

So far, studies of aggregation mechanisms have been made with the help of turbidity, solubility and sedimentation measurements. No observations have been made regarding the molecular details of this aggregation. A mechanism has been proposed for β-lg aggregation but, as mentioned above, no studies on the sizes or forms of these aggregates exist, more than that their sedimentation coefficient has been elucidated. The mechanism has been studied at neutral pH-values and at temperatures above 70°C. Here, interactions of reversible denatured molecules occur via irreversible steps to form a polydisperse set of aggregates. Studies with various techniques led to the discovery that two distinct group reactions can be separated. These are termed Type I and Type II aggregation. The reactions may be depicted as in formula 4 for β-lg. The D→A step represents the Type 1 aggregation and the
The reactions are sequential, with the large aggregates being formed at the expense of the smaller ones. Type I aggregation involves the establishment of intermolecular disulfide bonds observed with the help of starch electrophores and sedimentation velocity techniques (Sawyer, 1968), and tetramerization of 4 monomers has been suggested, partly based on the observations of a four-fold increase in particle weight (Briggs and Hull, 1945; Pantaloni, 1964). This sulfhydryl group aggregation occurs at temperatures above 70°C, also observed with the help of gel electrophoreses (Elfagm and Wheelock, 1978). During Type II aggregation, a much heavier component is formed, and this is "non-specific" without disulfide bond formation (Sawyer, 1968). Type II aggregation has also been further investigated with the help of electrophores technique (Briggs and Hull, 1945; Pantaloni, 1964; Elfagm and Wheelock, 1978).

The D→A₄ step represents the Type III aggregation. This step occurs when a sulfide blocking agent is added, for example, N-ethylmaleimide (NEM). It is described as a "non-specific" aggregation which occurs when Type I and Type II aggregations are blocked (Sawyer, 1968).

A study of the aggregation mechanism of whey protein at around pH 6-7 has been done by Morr and Josephson with the help of low- and high-speed centrifugation, Sephadex gel-filtration and polyacrylamide gel electrophores. They showed that the whey protein aggregation mechanism was almost identical with that of β-lg. The aggregation mechanism was divided into three reactions with different products and bonds. In the first step, thiol-disulfide group reactions were involved in the aggregation products. In the second step, intermediate sized protein aggregates were formed. Here, thiol-disulfide reactions were involved, and the reaction was also somewhat calcium-dependent. There is also a possibility that intermolecular hydrogen and hydrophobic bonding contribute to this second step in whey protein aggregation. The third step involves the formation of gross aggregate particles. This reaction is calcium-dependent (Kenkare et al, 1964; Morr and Josephson, 1968).
3.1.1 Temperature dependence

When considering the temperature dependence of Type I and Type II aggregations, some conflicts exist, see table 2.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Products formed</th>
<th>Sedimentation coefficient (S_{20w}) or MW (Da) of product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 60°C*240 min</td>
<td>A_n, A_1</td>
<td>15.2 S</td>
</tr>
<tr>
<td>99°C*10 min</td>
<td></td>
<td>5.6 S</td>
</tr>
<tr>
<td>2. 75°C*20 min</td>
<td>A_1, A_n</td>
<td>—</td>
</tr>
<tr>
<td>75°C*150 min</td>
<td>A_n</td>
<td>29.0 S</td>
</tr>
<tr>
<td>97°C*70 min</td>
<td>A_1</td>
<td>3.2 S</td>
</tr>
<tr>
<td>97°C<em>150 min 75°C</em>150 min +</td>
<td>A_1</td>
<td>3.7 S</td>
</tr>
<tr>
<td>NEM</td>
<td>A_t</td>
<td>2.3 S</td>
</tr>
<tr>
<td>NEM 97°C*150 min +</td>
<td>A_t</td>
<td>2.6 S</td>
</tr>
<tr>
<td>3. 76°C*20 min</td>
<td>A_1, A_n</td>
<td>2.9 S, 27.0 S</td>
</tr>
<tr>
<td>4. 74°C*20 min</td>
<td>A_1</td>
<td>—</td>
</tr>
<tr>
<td>77°C*20 min</td>
<td>A_1, A_n</td>
<td>—</td>
</tr>
<tr>
<td>80°C*20 min</td>
<td>A_n</td>
<td>15*10⁴ Da</td>
</tr>
<tr>
<td>85°C*20 min</td>
<td>A_n</td>
<td>20*10⁴ Da</td>
</tr>
<tr>
<td>5. 75°C*4 min</td>
<td>A_1</td>
<td>2.9 S</td>
</tr>
<tr>
<td>75°C*20 min</td>
<td>A_1, A_n</td>
<td>2.9 S, 32.8 S</td>
</tr>
<tr>
<td>85°C*150 min</td>
<td>A_n</td>
<td>38.6 S</td>
</tr>
<tr>
<td>90°C*90 min</td>
<td>A_n</td>
<td>39.5 S</td>
</tr>
<tr>
<td>95°C*60 min</td>
<td>A_n</td>
<td>43.7 S</td>
</tr>
</tbody>
</table>

Table 2: Some experimental data on the products of the thermodenaturation of β-lactoglobulin (Mulvihill and Donovan, 1987).

1 Briggs and Hull (1945), (pooled β-lactoglobulin, pH 6.9)
2 Sawyer (1968) (β-lactoglobulin A, pH 7.0)
3 Pantaloni (1964) (β-lactoglobulin A, pH 6.75)
4 Elfagn and Wheelock (1978) (commercial β-lactoglobulin, milk ultrafiltrate)
5 Sawyer et al (1971) (β-lactoglobulin A, pH 6.6)
According to Elfgang and Wheelock, the formation of large aggregates increased with temperature, as observed with the help of gel electrophoreses (Elfgang and Wheelock, 1978). Sawyer et al also found that the sedimentation coefficient of β-lg increased with increasing temperature, which was in accordance with the postulate that the degree of aggregation increases with increasing temperature (Sawyer et al, 1971). These findings conflict with the result of Briggs and Hull, who also studied the temperature dependence with the help of electrophoretic measurements. They suggest that Type I aggregation occurred above 65°C and reached a maximum between 75-85°C. Type II aggregation can proceed only after the first has occurred but proceeds at temperatures below those at which Type I is initiated. The result suggest that the smaller aggregates, A₁, are favoured at higher temperatures (Briggs and Hull, 1945). Data from Sawyer also suggest this temperature behaviour, i.e. at lower temperatures, Type II aggregation is favoured and the heavier form of β-lg, A₂, predominates (Sawyer, 1968). This was measured with the help of starch electrophoretic and sedimentation velocity techniques. This result is in agreement with the findings of Pantaloni (Pantaloni, 1964). The behaviour is not clear, and it has been suggested that at these temperatures some factors come into action which serve to block the conversion by decreasing the probability of mutual contact of the reactants (Briggs and Hull, 1945), or that some strongly exothermic reaction is the factor responsible (Pantaloni, 1964).

The methods used to study the temperature dependences of aggregation often involved examination of heated samples following cooling, or re-heating following cooling. The effects of this combined heat treatment on the material have not been considered, and further work is required in order to obtain agreement regarding the temperature dependence of Type I and Type II aggregation.
3.2 Environmental effects

3.2.1 Effect of pH

The aggregation, i.e. precipitation, is pH-dependent and varies over the whole pH-range. In figure 10, the influence of pH on the heat precipitation of whey proteins is depicted.

Fig.10 Effect of pH on the heat precipitation of whey proteins in acid whey. Heat treatment = 95°C for 5 minutes (Bernal and Jelen, 1985).

The transition from soluble to insoluble proteins takes place within a narrow pH-range. The protein precipitation is caused by heat treatment at 95°C for 5 minutes. The aggregation is favoured in the isoelectric pH-region. Here, the electrostatic repulsion is very low, and protein-protein interaction is likely to occur (Bernal and Jelen, 1985). Sulphhydryl oxidation or sulphhydryl-disulfide exchange reactions are also starting to take place after being negligible at lower pH-values (Watanabe and Klostermeyer, 1976).

At low pH-values, the denatured and undenatured protein remain in solution (Harwalkar, 1979). The molecules carry a net positive charge, which results in high electrostatic repulsion, and this reduces protein-protein attraction and subsequent aggregation. At this pH-value, the involvement of sulphhydryl oxidation or sulphhydryl-disulfide exchange reactions is expected to be negligible (Harwalkar, 1979; Shimada and Cheftel, 1988).
In the alkaline pH-range, the molecules carry a net negative charge and electrostatic repulsive forces dominate and restrain aggregation. At these alkaline pH-values, heat processing also caused a significant decrease in SH group and half-cysteine contents (Shimada and Cheftel, 1988).

Thus, the conditions which favour denaturation has the opposite effect on aggregation (Hermansson, 1979), i.e. greatest aggregation occurs in the isoelectric region and greatest denaturation occurs above and below the isoelectric region (see figure 9).

3.2.2 Effect of salts

The extent of whey protein aggregation is dependent on the presence of ions. It is a common and known phenomenon that ions induce aggregation, and that it is a concentration and pH-dependent process due to the fact that ions reduce the charge on the protein and cause isoelectric precipitation (Zittle et al. 1957). Effects of different ions have been studied, and it is clear that Ca$^{2+}$-ions have a greater effect on heat aggregation than do Na$^{+}$ and Mg$^{2+}$-ions (Varunsatian et al, 1983). Addition of other ions has also been investigated. For example, addition of FeCl$_3$ to whole whey reduced the solubility (Modler and Emmons, 1976).

3.2.2.1 Ca$^{2+}$-ions

The effect of Ca$^{2+}$-ions on the thermal behaviour of whey protein has been extensively researched, and added Ca$^{2+}$-ions induce aggregation (Hidalgo and Gamper, 1977). It has been elucidated that it is the formation of gross aggregate particles of whey protein and/or β-lg that is Ca$^{2+}$-dependent (Kenkare et al. 1964; Morr and Josephson, 1968). The whey protein that is most sensitive to Ca$^{2+}$ is β-lg (Varunsatian et al, 1983). In figure 11, the effect of Ca$^{2+}$-concentration on the aggregation of denatured β-lg is depicted as an effect on turbidity.

The whey protein seems to be less sensitive to Ca$^{2+}$-induced aggregation as the pH-value increases from 6.2 to 7.4, as shown in figure 11. The flocculation concentration is defined as the calcium concentration at which the transmittance is reduced to 50% of its initial value. These flocculation values are plotted in figure 12, curve A, and reveal a linear increase with increasing temperature. Curve B is calculated from the net negative charge on the β-lg molecule, obtained from its titration curve. It represents the equivalence between the amount of Ca$^{2+}$-ions and the negative charge on β-lg.
Fig. 11 Effect of Ca\(^{2+}\) concentration on the turbidity (light transmission at 600 nm) after a 10 min heat treatment at 80°C of a 1% β-lactoglobulin solution at different pH values. (The arrow indicates the level for determination of the flocculation value.) Temperature 25°C (calcium present during heat treatment) (De Wit, 1981).

Zittle et al have explained this coherence and proposed a role for Ca\(^{2+}\)-ions and pH in the thermocoagulation of β-Ig: precipitation of β-Ig in heated calcium-containing solutions is dependent on the net protein charge resulting from the binding of Ca\(^{2+}\) with certain specific groups on the protein, reducing the net negative charge to zero and causing isoelectric precipitation. Thus, isoelectric precipitation of heated β-Ig may be obtained by either lowering the pH to the isoelectric point or at the alkaline pH values by the addition of calcium. In the pH-range 6-8, the amount of calcium bound by β-Ig is equivalent to the net negative charge (Zittle et al, 1957).

In figure 12, the effect of adding Ca\(^{2+}\) after the heat treatment and subsequent cooling is also depicted, curve C. The flocculation values for curve C are determined according to the same procedure as those for curve A.
Fig. 12 Effect of pH on the calcium flocculation value of a 1% β-lactoglobulin solution, heated for 10 min at 80°C: a) when calcium is present during heat treatment (A); b) when calcium is added after heat treatment (C,D); c) when calculated from the net charge on the β-lactoglobulin molecule (B). Temperature 25°C (De Wit, 1981).

The figure clearly shows that they are three to four times higher than those in curve A (De Wit and Klarenbeek, 1984; De Wit, 1981). It has been suggested that some renaturation or rehydration of the protein at 25°C might be responsible for this increased stability (De Wit, 1981). From curve C, it can also be concluded that the sensitivity to calcium flocculation of heated β-lg is increased above pH 6.5. In curve D, NEM is added prior to heating to reduce the thiol activity. Calcium is added after heat treatment. It can be seen that a restored linear relation between the calcium flocculation value and the pH between 6.0 and 7.6 is achieved. This proves that during heat treatments an increased thiol reactivity sensitizes β-lg to calcium flocculation (De Wit, 1981; De Wit and Klarenbeek, 1984). The calcium content of
acid whey is different from that of rennet casein or cheese whey. The acid whey may contain 2.5-3 times more calcium, and this can be the reason for the difference in response to heat.

3.2.2.2 Na⁺-ions
The effect of NaCl on the heat denaturation and aggregation mechanism has also been investigated. Harwalkar and Kalab showed that low NaCl concentration, i.e. 0.05-0.1M, had a protective effect on β-lg compared to solutions free of salt. They also explain the stabilizing effect as a result of increased hydration. Stability may arise from the withdrawal of hydrophobic groups of protein from water, leading to a compact form (Harwalkar and Kalab, 1985a). At higher ionic strengths the rate of aggregation increased, and this is explained as the screening of electric charge by the ions leading to isoelectric precipitation, the same phenomenon as for Ca²⁺. Harwalkar and Kalab also studied the NaCl effect on β-lg aggregation and showed that the nature of the aggregation products depends on the forces which permit interaction between protein molecules (Harwalkar and Kalab, 1985b). Hermansson studied the NaCl effect on whey protein aggregation and showed that the presence of salt promoted aggregation. This due to reduction of the diffuse part of the electric double layer (Hermansson, 1979).
4. EMULSIONS

An emulsion is a two-phase system containing two immiscible liquid phases, one dispersed in the other. It contains high energy and is unstable. In order to form an emulsion, work is applied on two liquids. This will increase the interfacial area between the two liquids. It will also increase interfacial energy, since the state of free energy of the system increases with the increase in area of contact between the two liquids. As the system tries to keep the energy at a minimum, the dispersed phase forms spherical particles. This is one way to minimize the interfacial area. There is also a tendency in the system to minimize the energy by letting the droplets merge and coalesce. This is because large spheres have a smaller ratio of surface to volume than smaller ones, and the interfacial area will then decrease and, consequently, the energy as well (Mangino, 1984). If enough coalescence, the system will phase separate and create two phases. In this state the free energy is minimized as the area of contact between the two liquids is minimized.

The emulsion may be stabilized against coalescence, creaming, flocculation, phase separation and other destabilizing mechanisms if another component is added that is partially soluble in both phases. Such a component is called an emulsifier, and it is amphiphilic, i.e., it contains both hydrophobic and hydrophilic areas. An emulsion made of oil, water and an emulsifier still has a high energy, and it is generally said that it is thermodynamically unstable and will break, sooner or later (Mangino, 1984).

The most common emulsifiers in food products are proteins, since they are both amphiphilic and edible (Tornberg, 1990; Nakai, 1983; Phillips, 1981). They consist of amino acids, which are hydrophobic, neutral and hydrophilic. This is the reason for their surface activity, i.e. they orient themselves at a surface, for example, an oil-water surface, with the hydrophobic amino acids turning towards the oil-phase and the hydrophilic amino acids turning towards the water-phase (Tornberg, 1990; Graham and Phillips, 1979; Cheftel et al, 1985).

Emulsifiers adsorb at and coat an interface, providing stability to different food emulsions in various ways. For example, by lowering the interfacial tension of the emulsion they form an interfacial film with special properties etc. The surface activity of whey proteins and their long-time stabilizing power in emulsions have been investigated (Mangino, 1984; Yamauchi et al, 1980; Tornberg, 1990; Klemaszewski and Kinsella, 1991; Leman and Kinsella, 1989; Krog et al, 1989; Tornberg, 1978a; Voutsinas et al, 1983; Kella et al, 1989). The effect of different emulsifying processes is also of interest and has been studied (Tornberg and Hermansson, 1977; Tornberg,
1978b and 1978c). The emulsifying power of whey protein may be attributed to its amphiphilic nature, and, particularly, to its ordered globular structure, which on unfolding at the interface give rise to a tightly-packed viscoelastic film (Dickinson et al. 1989a).

In this chapter, emulsifying properties of food proteins, specially whey proteins, will be discussed from a scientific point of view. The surface forces involved, the theory and kinetics of adsorption, interfacial behaviour, the stability provided and factors affecting this stability as well as classification, will be among the topics dealt with.
4.1 Adsorption at interfaces

4.1.1 Theory of adsorption

Many different factors determine how rapidly a protein reaches and adsorbs at an interface, for example, solubility, the hydrophobic/hydrophilic balance, flexibility, size, net charge and other structural properties of the protein. These factors partially determine the initial emulsion characteristics (Klemazewski et al., 1990).

The protein is greatly hydrated in the aqueous solution and, as it approaches an interface, the charged groups of the protein will be less hydrated. This is energetically unfavourable, and the groups will be repelled from the interface. However, if they occur in an area of the protein with some flexibility, the structure of the protein will be altered. This may result in exposure of hydrophobic groups, previously buried in the interior of the molecule. These groups will orient themselves towards the hydrophobic phase in the emulsion, thereby lowering the interfacial tension. This behaviour is shown in figure 13. As the protein is adsorbed, it will partly unfold and fluctuate and also spread out in order to minimize the energy of the system. This will induce new surfaces, and it will continue until fluctuations and unfoldings of the protein structure no longer yield a conformation with lower energy (Phillips, 1981; Graham and Phillips, 1979; Mangino, 1984).

Partly denatured protein molecules may be more flexible and expose more hydrophobic groups than native molecules do. This will facilitate the adsorption at interfaces and decrease interfacial energy more rapidly. It seems that the proteins become more surface active on partial denaturation (Arnebrant et al. in press). Clearly, any factor or mechanism that induces this behaviour in proteins is positive and help to form and preserve the emulsion. The denaturation behaviour of proteins, specially whey proteins, has been discussed in chapter 2.
Fig. 13 Schematic conformation of a protein at an interface: (A), globular native protein immersed in an aqueous solution; (B), globular protein close to the interface; (C), adsorbed, unfolded and hydrated protein molecule (Cheftel et al, 1985).
4.1.2 Interfacial behaviour

It is important to know how proteins act at an interface in order to elucidate their emulsifying properties. They are macromolecules which adsorb and unfold at an interface to an extent determined by minimization of the interfacial energy. The degree of unfolding will also be determined by the surface area present relative to the amount of protein available, and by the flexibility of the protein. If the oil volume fraction is large and the protein concentration low, the degree of unfolding will be as high as possible in order to cover maximum surface area. On the other hand, if there is a high protein concentration, the unfolding will not be as complete (Phillips, 1981; Graham and Phillips, 1979; Klemaszewski et al, 1990).

The primary layer of adsorbed protein is irreversibly adsorbed. It has, for example, been proved for BSA that 3.3 mg m\(^{-2}\) provides a saturated monolayer with a film thickness of about 50-60 Å. After this monolayer formation, protein adsorption continues via multilayer formation of reversibly adsorbed molecules, which give rise to a coverage of 5-10 mg m\(^{-2}\) and a film thickness greater than 100 Å. The reversibly adsorbed multilayer molecules cause no change in the pressure, although the film thickness may increase to more than 100 Å (Graham and Phillips, 1979).

Interfacial behaviour is usually measured by studying the behaviour of the interfacial tension as a function of time, which decreases as the emulsion is formed, or the interfacial pressure as a function of time, which increases during the emulsion formation.

In figure 14, the interfacial tension decay is depicted as a function of time, as whey protein adsorbs at an soya oil-water interface. The arrows in the figure show the kinetic behaviour, and this will be described later on in section 4.1.4. This was measured with the help of the drop volume principle (Tornberg, 1977). This technique is rapid as the surface behaviour solutions come to equilibrium quickly. Measurements are performed by mounting a glass syringe vertically and allowing a drop of solution form and detach from the tip of the syringe. The time required for the surface tension to decrease, allowing the drop to detach, is measured. Interfacial behaviour has been widely studied, and whey proteins have been broadly investigated in this area (Tornberg et al. 1982; Tornberg, 1978a; Elizalde et al, 1991; Tornberg, 1990; Paulsson and Dejmek, 1992). Some of these studies will be reviewed below.
Fig. 14 The decay of interfacial tension of whey protein concentrate, suspended in 0.2M NaCl solution at pH 7, adsorbing at the soy bean oil-water interface depicted as a function of time \(1/2\) at different subphase concentrations: ○, \(1\times10^{-3}\); ●, \(1\times10^{-2}\); △, \(6.4\times10^{-2}\); ▲, 1; and □, 10 g kg\(^{-1}\) (Tornberg et al., 1982).

The properties of adsorbed and spread casein and whey protein films formed at an air-water interface in various ways have been compared. The relationship between surface concentration and surface pressure was investigated by reducing the area occupied by a fixed mass of protein to obtain a pressure-area (Π-A) isotherm. By spreading an increasing amount of protein at constant area, a pressure-concentration (Π-c) isotherm was also obtained. The interfacial pressure was measured using a Wilhelmy plate. In the Wilhelmy plate method, the change in the force required to maintain a plate at a constant immersion in a solution as the surface tension alters is continually measured with time. The surface pressure is obtained as the difference between the surface tension of pure solvent and the surface tension of the solution with the surface active component (Paulsson, 1990). It was concluded that Π-A isotherms are generally unaffected by changes in the structure of the spread molecule, while Π-c isotherms are sensitive to changes in protein structure. The study suggests that disordered proteins are more surface-active than globular proteins, agreeing with the discussion above about the flexibility of molecules enhancing emulsion formation. Π-c spread films and the surface pressure characteristics of the adsorbed films were proved to be similar, in that both Π-c isotherms and adsorption behaviour reflect the
magnitude of the forces preventing the unfolding of the molecule at the surface and can be used to provide information about protein structure (Mitchell et al. 1970).

The concentration dependence of the interfacial tension behaviour, obtained at the air-water interface, is followed in figure 15 with the help of the drop volume technique, described above. The concentration effects of soya protein, whey protein concentrate and caseinat were investigated at pH 7, both in distilled water denoted (0-7) and in 0.2 M NaCl-solution denoted (0.2-7). In the figure, \( \Pi_{40\text{min}} \) (surface pressure attained after 40 minutes) is plotted against the initial subphase concentration. Three regions may be elucidated in the figure: the high concentration range between \( 10^0 \) and \( 10^1 \) wt %, the medium concentration range from \( 10^1 \) up to and including \( 10^3 \) wt % and the low concentration range below \( 10^3 \) wt %.

![Fig. 15](image)

**Fig. 15** The surface pressure attained after 40 min, \( \Pi_{40\text{min}} \), as a function of the initial subphase concentration for all the proteins studied at different ionic strength. ●—●, soya protein (0.2-7); ○—○, soya protein (0-7); ✶—✶, whey protein concentrate (0.2-7); △—△, whey protein concentrate (0-7); ■—■, caseinat (0.2-7); □—□, caseinat (0-7) (Tornberg, 1978a).

In the high concentration range, the proteins are almost equal, independently of the conditions prevailing. In contrast, at medium concentration, differences in surface behaviour become evident. The caseinate (0.2-7) system proves to be the most effective interfacial tension depressor here and is, more or less, independent of
concentration. The opposite behaviour may be observed for the soya proteins, which show a low capacity for interfacial tension depression. The whey protein concentrate curves are in between those of caseinate (0.2-7) and the soya protein, but the addition of salt will increase their surface activity. At low concentrations, the whey protein concentrates lose their surface activity less abruptly than the caseinates. The soya proteins have no surface activity in this concentration range either (Tornberg, 1978a).

The surface film pressure (π) of β-lg, α-lα and BSA has been studied both in simulated milk ultrafiltrate (SMUF) and in water. The concentrations ranged from $10^{-6}$ up to 1% (w/v), and the times for the investigation varied from 30 s to 14 hr. It was found that in SMUF, at low concentrations, β-lg showed the highest surface activity, while at high concentrations β-lg and α-lα showed similar behaviour. BSA showed low surface activity. As pH approached the iso-electric point, π increased. For BSA this increase was faster than for the other proteins studied. It was also concluded that π in water was lower than in SMUF (Paulsson and Dejmek, 1992). The methods used for this study were the Wilhelmy plate and drop volume techniques, described above.

Some investigations have attempted to elucidate whether the adsorption of protein differs according to the nature of the interface, i.e. whether it is air-water or oil-water. A study regarding this was performed on β-casein, BSA and lysozyme. It was concluded that no drastic change occurred in interfacial behaviour when changing from air-water interfaces to oil-water interfaces (Graham and Phillips, 1979). This was also the case for a soya protein isolate, a sodium caseinate and a whey protein concentrate. It was, however, concluded that at a high protein concentration the surface activity was higher at an air-water interface than at an oil-water interface, whereas the reverse was found in the low concentration range. Suggestions were made about the proteins being less folded at the soya bean oil-water interface than at the air-water interface, since the adsorption process is more diffusion-controlled at the air-water interface. In oil-water systems where proteins unfold at the interface, the organic liquid more readily solvates the hydrophobic amino acid residues than air. This causes the organic liquid to penetrate the hydrophobic chains in the hydrophobic portions of the molecules and eliminate the non-polar interactions. Therefore, the probability of a more expanded form of the protein molecule is higher at the oil-water interface than at the air-water interface (Tornberg et al, 1982).
4.1.3 Adsorption at metal faces

Proteins have the ability to adsorb at most interfaces. Sometimes this is a desirable and essential reaction, for example, for protein function in biological systems, and sometimes it involves problems. The solution behaviour of the molecules strongly influences the adsorption. This aspect has more or less been neglected and the interest has been focused on the interfacial aspects of protein adsorption (Nylander, 1987).

Investigations have specially been performed on the adsorption of proteins at metal-water interfaces during heating, as this adsorption is referred to as a big problem in industry. The phenomenon is usually called fouling and results in cleaning problems, reduces the availability of the process equipment and increases processing costs. Proteins are heat sensitive and abundant in food. Unit operations involving heat are frequently used in the dairy industry, so that investigations in order to minimize or eliminate the fouling problem are essential. The role of whey proteins in fouling problems has been investigated and will be discussed here. A common method to measure fouling is by ellipsometry (Nylander, 1987).

Hegg et al (1985) studied the conditions under which globular proteins adsorb to a metal surface in a static system. The fouling of whey proteins, β-lg and α-la, on stainless steel was examined as a function of pH, ionic strength and temperature. It was found that the maximum adsorption of β-lg on stainless steel was reached at 120°C and at a value of 3 g protein/m². The extent of fouling was measured by difference in weight.

The adsorption of α-la and β-lg on hydrophilic chromium surfaces, using ellipsometry, has been investigated. The samples were heated at temperatures up to and exceeding the thermal denaturation temperature of the proteins, thus denaturing the proteins. It was found that, during this temperature treatment, denaturation of β-lg was irreversible, whereas the denaturation of α-la was highly reversible, as pointed out in chapter 2 in this review. It was shown that, when β-lg approaches its denaturation temperature, an aggregation occurs at the surface, starting after a time lag of several minutes. This was not found for α-la. Preheated solutions of β-lg had nearly the same adsorption behaviour at 25°C as the native protein, while the thermally denatured form of α-la seems to be more surface-active than the native form (Arnebrant et al, in press).

The competitive adsorption of β-lg and κ-casein on different treated chromium surfaces, both hydrophilic and hydrophobic, were investigated using ellipsometri. The
adsorption was followed with the help of $^{14}$C-labeled $\beta$-lg. The effect of a monolayer on the subsequent adsorption of the other protein was studied. The result shows that, when $\kappa$-casein is injected first, the adsorbed mass is very extensive and does not seem to be affected by injection of $\beta$-lg. On the other hand, when $\beta$-lg is adsorbed first, the adsorbed amount increases upon further injection of $\kappa$-casein. The experiments indicate that $\beta$-lg remains on the surface and $\kappa$-casein adsorbs at the already adsorbed $\beta$-lg layer (Arnebrant and Nylander, 1986). A simultaneous addition of the proteins reveals that both the composition of the adsorbed layer and the total amount of protein adsorbed are influenced by the character of the surface.

4.1.4 Kinetics of adsorption

It has been suggested that the adsorption of proteins to oil-water phases has two kinetic regions and that it depends upon the flexibility of the native protein (Krog et al, 1989; Phillips, 1981; Graham and Phillips, 1979). First, the initial adsorption is supposed to be diffusion-controlled, which results in a decrease in surface tension and an increase in $\Pi$ and in protein load. Secondly, after an initial protein film has been formed, a penetration-controlled adsorption takes place, which may result when $\Pi$ increases at a constant protein load. This is due to the protein molecules creating space in the existing film by penetration, rearrangement or by changing their conformation, and by fluctuations at the surface.

The kinetics of adsorption have been described with the help of relaxation times, $\tau_1$ and $\tau_2$. $\tau_1$ describes the adsorption when both interfacial pressure and protein load ($\Gamma$, mg protein adsorbed/m$^2$ fat) are increasing. $\tau_2$ is usually in the range of 2 hr when $\Gamma$ is around 2 mg m$^2$. $\tau_2$ relates to the situation where surface pressure increases at constant $\Gamma$ due to the rearrangement of protein molecules at the surface (Phillips, 1981; Graham and Phillips, 1979).

The kinetics of protein adsorption at an interface can be studied by monitoring surface concentration, surface pressure and interfacial tension decay (Leman and Kinsella, 1989). The rate of reduction of interfacial tension is usually explained by dividing the behaviour into three consecutive or concurrent processes, correlating exactly with the two-step process described above: the diffusion of whole protein molecules or aggregates to and attachment at the interface; spreading or unfolding of already adsorbed molecules on the interface; molecular rearrangements and reconformations of adsorbed molecules (Tornberg et al, 1978a; Mitchell et al, 1970; MacRitchie and Alexander, 1963).
Tornberg (1978a) studied the adsorption behaviour of three food proteins, a soya protein isolate, a sodium caseinate and a whey protein concentrate, at the air-water interface with a drop volume method described above. The kinetics of surface tension decay were evaluated in terms of different ionic strengths and concentrations. It was concluded that whey protein diffuses quickly to an interface compared to the other proteins studied. This was explained by their aqueous association, which mainly consists of small molecular complexes. In table 3 the kinetics of a whey protein concentrate are depicted as a function of ionic strengths and concentration. It may be concluded from the table that diffusion is slower and spreading easier in distilled water than in 0.2M NaCl-solution. As the concentration decreases, the effects noticed were that the diffusion became slower, the penetration step starts at a lower $\Pi$ and $\Delta A_{ag}$ is larger at the same $\Pi$. These effects were also noted for the soya protein isolate (Tornberg, 1978a).

<table>
<thead>
<tr>
<th>Type of protein system</th>
<th>Conc. % (w/w)</th>
<th>Rate-determining step</th>
<th>$\Pi_{tr}$</th>
<th>$\Pi_A$</th>
<th>$\Delta A$/ag (nm$^2$)</th>
<th>$\Delta \Pi_{ag}$ (%)</th>
<th>$\Pi_{ag}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.2-7)</td>
<td>$10^5$</td>
<td>Diffusion</td>
<td>15.6</td>
<td>17.4</td>
<td>1</td>
<td>63</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penetration</td>
<td>25.1</td>
<td>2.6</td>
<td>57</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rearrangement</td>
<td>26.2</td>
<td></td>
<td>42</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(0.2-7)</td>
<td>$10^{-1}$</td>
<td>Diffusion</td>
<td>9.5</td>
<td>8.5</td>
<td>2</td>
<td>43</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penetration</td>
<td>20.3</td>
<td>1.7</td>
<td>68</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penetration</td>
<td>20.8</td>
<td>7.0</td>
<td>30</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(0.2-7)</td>
<td>$3 \times 10^{-1}$</td>
<td>The effect of surface enlargement</td>
<td>1.7</td>
<td>1.7</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>(0.2-7)</td>
<td>$1 \times 10^{-1}$</td>
<td>The effect of surface enlargement</td>
<td>0.9</td>
<td>0.5</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>(0-7)</td>
<td>$10^9$</td>
<td>Diffusion</td>
<td>15.5</td>
<td>15.3</td>
<td>2</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penetration</td>
<td>25.4</td>
<td>1.6</td>
<td>37</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penetration</td>
<td>26.7</td>
<td>6.1</td>
<td>61</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>(0-7)</td>
<td>$10^{-1}$</td>
<td>Diffusion</td>
<td>12.6</td>
<td>11.6</td>
<td>8</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penetration</td>
<td>22.1</td>
<td>1.3</td>
<td>92</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>(0-7)</td>
<td>$3 \times 10^{-1}$</td>
<td>The effect of surface enlargement</td>
<td>1.9</td>
<td>2.5</td>
<td>10</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>(0-7)</td>
<td>$1 \times 10^{-1}$</td>
<td>The effect of surface enlargement</td>
<td>9.5</td>
<td>8.9</td>
<td>16</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Parameters describing the kinetics of the lowering of interfacial tension at the air-water interface of whey protein concentrate dispersions at different ionic strengths and concentrations (Tornberg, 1978a).

In table 3, $\Pi_{ag}$ is the attained surface pressure, $\Pi_{tr}$ is the surface pressure attained during the "diffusion" step, $\Delta A_{ag}$ the area in the surface film which adsorb an active
group (ag), $\Delta t_{ag}/40$ and $\Delta \Pi_{ag}/\Pi_{ag}$ indicate, respectively, the relative contribution of the time elapsed and $\Pi_{ag}$ of each rate-determining step in relation to 40 min and to the surface pressure attained after 40 min.

The kinetics of adsorption of a soya protein isolate, a sodium caseinate and a whey protein concentrate have been investigated at an oil-water interface, using the drop volume method, and compared with those at an air-water interface. The ranking order with respect to the surface activity was the same at both the air-water interface and the oil-water interface. The kinetics are depicted in figure 14, where the interfacial tension is monitored against time$^{12}$ for a whey protein concentrate adsorbing at a soya bean oil-water interface. The process is divided into three consecutive processes. The maximal slopes, indicated as linear parts of the plot by arrows, are thought to be diffusion-controlled and are the rate-determining step at this time of the process. After this linear part, the rate of surface tension decrease declines and a new linear relationship is observed. A high adsorption energy barrier exists here and diffusion will no longer be the rate determining step, but the rate of protein penetration into the surface film will. The barrier consists of the work done in creating an area in a surface film of surface pressure $\Pi$ in order to adsorb an active group into the protein molecule. At the end of the adsorption process, i.e. no areas are created in order to adsorb protein segments, a rearrangement process takes place (Tornberg et al., 1982).

4.1.5 Selectivity in adsorption

The adsorption of whey protein has proved to be selective and influenced by pH and temperature (Yamauchi et al., 1980). As the protein adsorbs at the interface, it initially involves hydrophobic interaction between hydrophobic groups of the protein and the hydrophobic phase constituting the emulsion, for example, the oil phase. This may be one of the reasons for the selective adsorption noticed, i.e., it reflects the difference in relative abundance of hydrophobic groups in the proteins. Another important factor affecting the selectivity is the iso-electric pH-value of the protein. At this pH-value the repulsive forces between proteins are low and allow rapid adsorption and association, leading to the formation of an interfacial film (Leman and Kinsella, 1989).

The selectivity of adsorbed whey protein has been studied by sodium dodecylsulfate-polyacrylamide gel electrophoresis. The investigation was performed in the pH-range 3-9, and the results are shown in table 4 below. It may be noted from the table that marked selectivity in adsorption of individual proteins is observed at any of the pH-values studied. No correlation between the adsorbability and surface hydrophobicity
was observed in this study. However, it was pointed out that the adsorption of monomolecular proteins to fat surfaces must depend upon the effective hydrophobicity of the proteins. At pH 5, the iso-electric pH-value for whey protein, the amount of adsorbed protein proved to be higher than at any other pH value. This reflects the fact that low repulsion exists between the proteins. It was also pointed out in the article that conformational properties of whey proteins, such as the flexibility of the structure, are important in the adsorption and may, to some degree, be responsible for the selectivity (Shimizu et al, 1981).

<table>
<thead>
<tr>
<th>pH</th>
<th>Whey proteins (%)</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-la</td>
<td>48.3</td>
<td>24.4</td>
<td>11.0</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>β-lg</td>
<td>12.9</td>
<td>16.6</td>
<td>46.1</td>
<td>61.6</td>
<td></td>
</tr>
<tr>
<td>Casein+Ig, light chain</td>
<td>31.8</td>
<td>33.0</td>
<td>27.9</td>
<td>18.6</td>
<td></td>
</tr>
<tr>
<td>Ig, heavy chain</td>
<td>2.3</td>
<td>7.0</td>
<td>3.4</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Serum albumin</td>
<td>1.0</td>
<td>10.6</td>
<td>2.7</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Transferrin+lactoferrin</td>
<td>3.7</td>
<td>8.4</td>
<td>8.9</td>
<td>7.4</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Relative abundance of individual whey proteins in the protein fraction absorbed on the fat globule surface of emulsions at various pH values (Shimizu et al, 1981).

* The whole whey protein fraction used in the present experiment contained a small amount of casein.

b SDS-PAGE gels were stained with comassie blue R-250 and scanned at 550 nm by using a Gelman DCD-16 densitometer.

Yamauchi et al (1980) studied the emulsifying properties of whey proteins over the pH range 3-9. They had already concluded, that the amount of protein adsorbed was highest at pH 5 and that the protein film was heterogeneous, whereas at pH 9 the film consisted mainly of β-lg. The protein structure at the interfacial film was found to be different at different pH-values. Around pH 5, the proteins adapt a granular structure. This may reflect a more aggregated form of the protein at the interface. In contrast, around pH 9, a thinner film is formed reflecting a more ordered orientation of the proteins at the interface. The results obtained in this study reflect the importance of the electrostatic nature and of the conformation of the proteins in the adsorption at the fat-water surface.

It has also been proved that time-dependent changes take place in the composition
of the film at the oil-water interface. Dickinson et al (1989a) studied the effect of whey proteins to displace one another at an interface in an emulsion. It was concluded that the exchange reactions existed but they were slow and limited in extent for β-lg and α-la. It was also concluded that β-lg was more difficult to displace than α-la.
4.2 Surface forces

Surface forces are static forces acting between particles. They depend on the particle separation and are influenced by the properties of both the emulsion droplet surface and the separating medium. They include van der Waals, electrostatic double layer, hydration, hydrophobic and steric forces (Bergenståhl and Claesson, 1990) and they aid both the stabilization and destabilization mechanisms of the emulsion. However, in the right combination and under the right conditions, they may aid stabilization.

The DLVO-theory (Derjaguin-Landau-Verwey-Overbeek, named from the initials of its principal authors) describes colloidal stability and is often applied to emulsions. It states that dispersed particles are subjected to two independent forces, van der Waals force of attraction and electrostatic force of repulsion arising from the presence of an electrical double-layer at the particle surface. The net interaction is the sum of these two forces. Thus, if the repulsion potential exceeds the attraction potential, an energy barrier opposing collision results. If the magnitude of this energy barrier exceeds the kinetic energy of the particles, the suspension is stable. If the distance between the particles is small, the van der Waals force is significant, whereas at intermediate distances the repulsive potential, i.e. electrostatic double-layer force, is larger than the attraction potential (Nawar, 1985). However, double-layer forces are not very important in food emulsions, hence, the DLVO-theory can rarely explain emulsion stability or solve stability problems in food systems, as it does not take all forces involved in emulsion stability into consideration (Bergenståhl and Claesson, 1990).

Electrostatic double-layer forces are repulsive forces generated when two identically charged surfaces approach each other in an electrolyte solution. This will induce clustering of ions from the solution of opposite charge nearby and thus form an ionic atmosphere. This repulsive force can be explained to be caused by an increased osmotic pressure at the midpoint between the surfaces (Bergenståhl and Claesson, 1990). Two regions of charge may be distinguished here. First, there is a dense immobile layer of ions that stick tightly to the surface of colloidal particles. This layer may also include water molecules, if that is the support medium. The charged unit attracts an oppositely charged ionic atmosphere. The inner shell of charge and the outer atmosphere are called the electrostatic double layer. The primary role of the electrostatic double layer is to confer kinetic stability (Atkins, 1987).

Van der Waals forces are those forces obtained between permanent and induced dipoles and other multipoles on molecules and particles. They are small, and the
nature of the interaction is related to the distance between the atoms and/or molecules (Israelachvili, 1985). The van der Waals forces are more discussed in section 5.2.1.

Hydrophobic molecules, i.e. hydrocarbon, in aqueous solution, give rise to the association of water molecules into clathrate like structures. These structures minimize the free energy of the system, and make the unfavourable water/hydrocarbon contact less unfavourable than it would have been without such structures. Hydrophobic interactions are the association of apolar groups in an aqueous environment. This association decreases the area towards the aqueous solution and, since the exposure of non-polar groups to water is thermodynamically unfavourable, the hydrophobic interaction is encouraged. The interaction is attractive, and the driving force is removal of hydrophobic groups from water (Fennema, 1985).

There is a tendency to hydrate the polar head groups at an interface, while, at the same time, keeping the non-polar chains together. On removal of water molecules, solvating these polar head groups, a repulsive force is measurable called the repulsive hydration force. This force have been measured with different techniques and a review of this is given by Bergenstahl and Claesson (1990).

4.2.1 Interactions due to adsorption of polymers

The presence of different polymers, specially emulsifiers, in the solution and/or on the emulsion droplet surface may affect the forces acting between the droplets. This will be treated in this section, and the discussion is largely based on that of Bergenstahl and Claesson (1990). The polymers may adsorb to the surface irreversibly, reversibly or not at all. This may affect the forces in different ways.

4.2.1.1 Irreversibly adsorbed polymers

When the polymer adsorption/desorption rate is slow compared with how fast the polymer coated droplets approach each other, it is generally said that the polymer is irreversibly adsorbed to the droplets. Models describing the interaction between particles carrying these irreversibly adsorbed polymers have been developed (de Gennes, 1984, 1987; Scheutjens and Fleer, 1980, 1985; Fleer and Scheutjens, 1986). The system is not in equilibrium with the bulk solution, and the total amount of polymers, irreversibly adsorbed on the surfaces, is independent of the surface separation. Thus, the situation is referred to as a quasiequilibrium state, as it is often assumed that the speed of approach is sufficiently slow for the irreversibly adsorbed polymers to adopt the most favourable conformation for each surface separation. Hence, there is an equilibrium within the layer.
As the polymers have adsorbed irreversibly, the forces are affected by the polymer/solvent interactions, surface coverage and polymer concentration. These factors determine the sign, range and magnitude of the polymer-induced forces. Adsorbed polymers may generate interparticle forces by bridging from one surface to the other or through interacting polymer segments. Bridging is significant when the surface coverage and the polymer concentration are low or the time for adsorption is short. An attraction is obtained at large separations due to bridging. Force contributions arising from polymer/polymer interactions become more important as the surface coverage increases and at the same time, bridging polymers become less important. Such force contributions are:

- An ideal entropy of mixing between segments
- Changes in solution of polymer segments
- Volume exclusion effects that decrease the number of possible conformations and give rise to a true steric repulsion
- Electrostatic effects

The first and third contribution always become increasingly repulsive as the surface separation is decreased. The second contribution may be either repulsive or attractive. The fourth contribution is always repulsive in monovalent electrolytes, but might be attractive due to correlation forces if divalent cations are present.

4.2.1.2 Reversibly adsorbed polymers
Under conditions where polymers adsorb reversibly, full equilibrium is assumed to exist and purely attractive forces are generated. This is because, as some polymers desorb from the surfaces, a greater fraction of the remaining polymers will attach to both surfaces pulling them towards each other (Scheutjens and Fleer, 1985). The models described by de Gennes and Scheutjens and Fleer (se above) also describe the forces developed between surfaces carrying reversibly adsorbed polymers. No steric stabilization by adsorbed polymers, under full equilibrium conditions, can be obtained. But, due to a very slow desorption of the polymers, they are able to sterically stabilize emulsions, because they are retained on the surface during a collision event.
4.2.1.3 Non-adsorbing polymers

For steric reasons, the concentrations of polymer segments of non-adsorbing polymers are lower close to the surface than in the bulk solution. When polymer-depleted layers extend all the way between surfaces, an attractive osmotic force is generated, called depletion attraction. This may cause flocculation of larger particles and phase separation of smaller particles. It is strongly dependent on the polymer concentration (Sperry, 1984).
4.3 Emulsifying properties

The amphiphilic property, and thereby the emulsifying property, of a protein is principally determined by its hydrophobicity, solubility and flexibility and has been investigated by a number of scientists. The effects developed by these factors will be discussed below and, in connection with this, some investigations performed on whey proteins will also be treated. Worth mentioning is that the composition of whey proteins has also proved to affect the emulsifying properties, but as this is not so extensively investigated as the other factors, it will not be dealt with in a separate section (Kim et al., 1987).

4.3.1 Effect of hydrophobicity

Hydrophobicity has been investigated thoroughly, since the hydrophobic interactions are considered to play an important role in the functional properties of food proteins. In the early stages, the total hydrophobicity of a protein was measured as the sum of the side chain hydrophobicities of constituent amino acids. It has, however, now been made clear that the surface hydrophobicity, \( S_0 \), plays a much greater role in the emulsifying properties of proteins. A method for \( S_0 \) measurements has been proposed which involves using cis-parinaric acid as a fluorescence probe (Kato and Nakai, 1980). This method shows good correlations between \( S_0 \) and interfacial tension and the emulsifying activity of the proteins studied (Kato and Nakai, 1980; Kato et al., 1981).

The hydrophobic properties of whey proteins have been studied in order to elucidate and improve their emulsifying properties. This has been done by studying the effect of heat on whey protein samples (Mangino et al., 1987; Voutsinas et al., 1983). Usually, when a protein solution is heated, denaturation takes place. Whey proteins are globular proteins, and it is generally said that in their native state they fold themselves and bury their hydrophobic amino acids in the interior of the globule as this minimizes the free energy. The denaturation phenomenon, discussed in chapter 2 in this review, is linked to the unfolding of the molecule. This probably results in exposure of the hydrophobic groups on the protein and thereby an increase in the emulsifying properties may be obtained.

In table 5 the hydrophobicity of heated and non-heated whey protein and \( \beta \)-lg samples is compared. The table shows that, as protein denaturation of whey protein samples proceed, the \( S_0 \) first increases and then decreases as excessive heating takes
place. This is probably due to participation of some of the exposed hydrophobic
groups in hydrophobic interactions (Voutsinas et al., 1983; Nakai, 1983). Voutsinas
et al. (1983) also found, in agreement with the table below, that with increasing $S_o$ the
emulsifying properties initially increased and then decreased. This is explained by the
fact that the emulsifying properties depend on suitable balance between the
hydrophilic and lipophilic properties of the protein and do not necessarily increase
as the protein become more lipophilic (Aoki et al., 1981). One can imagine that a
small transference of the balance between the hydrophilic and lipophilic properties
may increase the emulsifying properties.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$S_o$</th>
<th>Solubility index (%)</th>
<th>EAI (m²/g)</th>
<th>ESP (min)</th>
<th>FBC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lg, control</td>
<td>426</td>
<td>100.0</td>
<td>96</td>
<td>27.20</td>
<td>4.2</td>
</tr>
<tr>
<td>β-lg, heated</td>
<td>192</td>
<td>6.4</td>
<td>51</td>
<td>25.50</td>
<td>16.5</td>
</tr>
<tr>
<td>Whey protein, control</td>
<td>182</td>
<td>88.7</td>
<td>87</td>
<td>50.30</td>
<td>74.5</td>
</tr>
<tr>
<td>I. pH 6.0, 80°C, 4 min.</td>
<td>211</td>
<td>75.2</td>
<td>98</td>
<td>61.30</td>
<td>75.2</td>
</tr>
<tr>
<td>II. pH 6.0, 80°C, 5 min.</td>
<td>164</td>
<td>65.5</td>
<td>89</td>
<td>63.00</td>
<td>72.5</td>
</tr>
<tr>
<td>III. pH 6.0, 80°C, 15 min.</td>
<td>128</td>
<td>50.4</td>
<td>82</td>
<td>48.30</td>
<td>100.8</td>
</tr>
<tr>
<td>IV. pH 5.8, 80°C, 15 min.</td>
<td>132</td>
<td>61.6</td>
<td>85</td>
<td>46.30</td>
<td>99.4</td>
</tr>
</tbody>
</table>

Table 5. Relationships between protein hydrophobicity ($S_o$), solubility index,
emulsifying activity (EAI), emulsion stability (ESI) and fat binding capacity (FBC)
of various proteins* (Voutsinas et al., 1983).

* I: 0.1 M NaCl added; II: NaCl not added
* average of duplicate determination

This argument suggests that partial denaturation, without coagulation, may be a good
alternative when attempting to obtain the highest emulsifying activity of a protein.
The denaturation chapter of this review shows that the denaturation of whey protein
is stepwise and does not have to involve complete unfolding of the protein. This
allows partial denaturation to take place under moderate heating conditions. The
effect of partial denaturation on ovalbumin and lysozyme has been studied and
proved to increase hydrophobicity as well as emulsifying properties (Kato et al.,
1981). From table 5 it is also concluded that, under moderate heating conditions, i.e.
conditions that are thought to partially denature the whey proteins, the emulsifying
conditions are optimal and decrease as the heat treatment becomes severe.
The solubility index, emulsion activity index (EAI), emulsion stability index (ESI) and fat binding capacity index (FBC) are included in the table. $S_o$, EAI and ESI correlate well in the table. EAI and ESI will be discussed in sections 4.4.2 and 4.4.3, respectively. The FBC is based on the concept that the phenomenon occurring at the oil-water interface is an adsorption one. It refers to the ability of the protein to take part in lipophilic interactions and to bind fat. Table 5 clearly shows that the FBC is improved upon by denaturation (Voutsinas et al, 1983). This may be related to the increase in exposure of hydrophobic groups. A simple turbidimetric method has been developed for determining FBC (Voutsinas and Nakai, 1983).

4.3.2 Effect of solubility

When a protein solution is heated, denaturation takes place and the solubility will usually be reduced (Voutsinas et al, 1983; Leman and Kinsella, 1989; Nakai, 1983). The solubility of whey proteins is an important property, governing their functional behaviour and potential application to food processing. Their solubility index can be seen in table 5. There has been disagreement regarding the solubility and emulsifying properties of proteins. A positive correlation between them has been reported (Crenwelge et al, 1974; Volkert and Klein, 1979; Yasumatsu et al, 1972; De Wit and De Boer, 1975) as well as a negative one (McWatters and Cherry, 1975; McWatters and Holmes, 1979a and b; Aoki et al, 1980; Wang and Kinsella, 1976). These observations have, largely, concerns soya proteins and whey proteins.

An explanation of the above discrepancy was made by Nakai (1983), who suggested that both hydrophobicity and solubility should be taken into consideration in order to explain the emulsifying phenomena of heat-denatured proteins. He based this assumption on the result from Voutsinas et al (1983), who concluded that $S_o$ is correlated with the emulsifying capacity of heat-denatured proteins. Under moderate heating conditions, the hydrophobicity is an important emulsifying factor rather than the solubility. As the heat denaturation progress, the solubility decreases and becomes an important controlling factor for emulsification, in addition to hydrophobicity.
4.3.3 Effect of flexibility

The emulsifying properties of a protein are also determined, as mentioned above, by its molecular flexibility. Increased flexibility enhances unfolding at the interface. This allows more favourable alignment of the hydrophobic and hydrophilic regions of the protein in the preferred phase (Graham and Phillips, 1979; Kato et al, 1985; Kato et al, 1986; Phillips, 1981; Klemaszewski et al, 1990). The operating conditions applied in order to form an emulsion are sensitive, as they may increase the flexibility of the protein thereby causing unfolding and exposure of hydrophobic groups to the non-aqueous phase.

Kato et al (1985) compared the emulsifying properties of BSA and lysozyme. It was concluded that BSA has good emulsifying properties, whereas lysozyme has poor. This difference may be due to the fact that BSA has higher surface hydrophobicity and a more flexible conformation (Kato et al, 1986). It was suggested that the surface hydrophobicity is a static factor and the flexibility a dynamic factor in the surface properties of the protein (Kato et al, 1985).

Many investigations have addressed protein flexibility in relation to the disulphide bonds in proteins. Proposals exist about them contributing to the rigidity of the protein and preventing complete protein unfolding at the interface. This results in less surface activity of the protein and suggests that disulphide bonds give poor emulsifying capacities (Graham and Phillips, 1979; Phillips, 1981). Cleavage of disulphide bonds in whey proteins has been investigated, and an improvement in the emulsifying properties by favourable changes in conformation, surface hydrophobicity, solubility, viscosity and folding has been observed (Klemaszewski et al, 1990; Klemaszewski and Kinsella, 1991; Kella et al, 1989; Kato et al, 1985).

Protein S-S bonds can be cleaved with the help of mild reducing agents, such as β-mercaptoethanol, DTT, etc, and then blocking the liberated free sulphhydryl groups with, for example iodoacetamide, since the sulphhydryl will probably oxidize back to the disulphide (Means and Feeney, 1970). A method for disulfide bond cleavage has been developed that is practical for food proteins. It involves sulfite, molecular oxygen and catalytic amounts of cupric ion (Kella and Kinsella, 1985). The cleavage reaction may be viewed as:

\[ \text{PS-S\text{\text{-}}SP+SO}_3^2\text{\text{-}}\text{PS-SO}_3^-+PS^- \]

and catalysed by Cu\textsuperscript{2+} and oxygen. The disulphide bonds are unsymmetrically cleaved and the reaction can be kinetically controlled. This makes it possible to regulate the degree of S-S bonds cleaved in a protein fraction.
Klemaszewski et al (1990) have, with this method, concluded that BSA increased its emulsifying property when sulfitolysis of disulphide bonds had occurred. They related this to the increasing flexibility, giving rise to exposure of normally hidden hydrophobic side groups of the protein.

Kella et al (1989) found, also with this method, that up to a disulphide bond cleavage of 50%, whey proteins unfold and aggregate with a concomitant increase in surface hydrophobicity. Above 50% bond cleavage, proteins disaggregate and unfold, and a decrease in surface hydrophobicity and adsorption is observed.

In contradiction to the results obtained by Kella et al (1989), Klemaszewski and Kinsella (1991) found that the emulsifying properties of whey protein isolate were improved as the degree of sulfitolysis increased from 0% to 100%. The sulfitolysis was performed according to the procedure discussed above.

The difference between the two investigations may arise from the fact that Kella et al (1989) measured on air-water systems, while Klemaszewski and Kinsella (1991) measured on oil-water systems containing different degrees of oil. A possible explanation, related to the discussion about difference in behaviour at air-water and oil-water interfaces in section 4.1.2, is that the probability of a more expanded form is higher at an oil-water interface than at an air-water interface because the hydrophobic groups are more readily dissolved in oil than in water. This suggests that more cleaved disulphide bonds and, by that, more unfolded, flexible proteins are preferred at an oil-water interface than at an air-water interface.
4.4 Characterization of emulsifying properties

The emulsifying properties of proteins are usually characterized by emulsion capacity (EC) or emulsion activity (EA), which reflect the ability of the proteins to aid the formation and stabilization of newly created emulsions, and emulsion stability (ES), which reflects the ability of the proteins to impart strength to emulsions for resistance to stress (Patel and Kilara, 1990). EC and EA are usually favoured by highly disordered and hydrophobic proteins, which are able to rapidly reduce the surface tension of newly formed emulsions. An example of a proper protein for this is β-casein. ES, on the other hand, is governed by a more ordered globular protein structure, which is able to form a tightly packed viscoelastic structure. β-Ig is an appropriate protein for ES (Kato and Nakai, 1980; Dickinson et al, 1989a; Dickinson et al, 1989c).

4.4.1 Emulsifying capacity

The EC denotes the maximum amount of oil emulsified under specified conditions by a standard amount of protein, i.e. EC is related to the interfacial area that can be coated by the protein (Pearce and Kinsella, 1978; Waniska et al, 1981). A simple method has been developed in order to determine EC (Swift et al, 1961). It involves adding oil to a protein dispersion under constant stirring. The procedure is terminated when a sudden drop in emulsion viscosity is indicated. This is the inversion point when a water-in-oil emulsion is formed from an oil-in-water emulsion. This method has been improved upon and is widely used. For example, the sudden drop in viscosity at the inversion point also results in a sharp drop in electrical current. Measuring this allows a precise determination of the inversion point (Crenwelge et al, 1974). The change in electrical resistance has also been used as an indication of the inversion point (Webb et al, 1970), as well as the addition of an oil-soluble dye, which facilitates the visual recognition of the phase inversion (Marshall et al, 1975; Wang and Kinsella, 1976).

Several parameters and variables affect the determination of EC. For example, the blender speed, i.e. higher blending speeds give smaller apparent EC (Swift et al, 1961; Carpenter and Saffle, 1964), the amount of soluble protein in the original aliquot used, the speed of mixing, the final temperature of the emulsion, the amount of oil initially added, different emulsification procedures, etc (Carpenter and Saffle, 1964; Waniska et al, 1981). The EC of leaf protein has, for example, proved to depend on protein concentration, its solubility, pH of the medium, sucrose and
sodium chloride additions (Wang and Kinsella, 1976). Thus, EC is not only a property of the protein studied, but is a property of the emulsion system, the equipment and method used to produce the emulsion (Tornberg and Hermansson, 1977). Furthermore, if the amount of oil is less than that required for phase inversion, it is not clear how EC is related to the amount of emulsifier required to produce a satisfactory emulsion. The EC value observed may also be incorrect if the emulsion formed is very viscous, due to inefficient or incomplete mixing of oil into the emulsion (Pearce and Kinsella, 1978). The capacity of available proteins to stabilize an emulsion by coating the interfacial area may be investigated, using an turbidometric technique (Ivey et al, 1970).

4.4.2 Emulsifying activity

The ability of proteins to aid in emulsion formation and stabilization of newly created emulsion is described as EA. Thus, a fixed oil phase volume is necessary for EA (Waniska et al, 1981). EA is usually measured by determining the particle size distribution of the dispersed phase by microscopy, Coulter counting or spectroturbidity (Walstra et al, 1969). Whichever procedure is chosen, an average diameter of the dispersed phase is determined, and from these data the interfacial area can be calculated. The microscopic techniques may be time-consuming and show poor reproducibility, whereas the Coulter counter method is not so time-consuming and more reproducible. The spectroturbidity method is also simple and rapid and theoretically sound (Walstra et al, 1969). The methods mentioned have both advantages and limitations, and in order to study EA, it is advisable to use more than one method to obtain reliable results.

The EA of BSA has been studied. It was proved, with the help of light microscopy and spectroturbidity, that EA decreased as the level of disulphide bonds in the protein decreased. Urea (8M) eliminated EA, while succinylation significantly enhanced EA for BSA (Waniska et al, 1981). In contrast, EAI (emulsion surface area per gram of protein) of WPI was improved as sulfitolysis of disulphide bonds occurred (Klemaszewski and Kinsella, 1991). The methods used to evaluate EAI were related to spectroturbidity (Walstra, 1968; Pearce and Kinsella, 1978). The difference in the results may be explained by the fact that different protein samples were used and that the emulsion preparation techniques were not the same.

Klemaszewski et al (1992) studied the EA of whey protein isolates, β-lg, α-la, BSA, casein, κ-casein and lysozyme. It was proved that EAI increased with increasing protein solubility and hydrophobicity. The methods used were spectro-turbidometric.
In table 5, EAI is shown for whey proteins. It is clearly depicted that EAI follows the surface hydrophobicity very well.

4.4.3 Emulsion stability

The capacity of proteins to stabilize emulsions during longer periods of time will be treated in this section. Proteins aid ES in many different ways, for example, by reducing the interfacial tension and the tendency of the dispersed droplets to merge each other and coalesce due to interparticle forces acting between droplets. This is specially pronounced if the emulsion possesses a net repulsive force. The forces involved are van der Waals attractive forces, electrostatic interactions, steric factors related to the surface active agents, osmotic effects, hydration forces, viscosity of the medium etc. These forces have already been discussed in section 4.2. In the right combination, they may generate an energy barrier against the close approach of individual droplets.

Fig.16 A schematic representation of emulsion instability (von Lange and Kurzendörfer, 1974).

ES is an important criterion in food applications and it has been investigated extensively under different conditions (Friberg et al, 1990; Leman and Kinsella, 1989). As mentioned before, an emulsion is thermodynamically unstable. The reason
for this is that the free energy of an emulsion is higher, due to the big interfacial area, than the energy of two liquid phases. (Leman and Kinsella, 1989; Mangino, 1984). Thus, an emulsion will break down, sooner or later, and current research effort is devoted to improve ES in order to extend the life time of the emulsion. This will give products with a longer shelf-life.

In order to improve ES, one has to minimize the breakdown mechanisms existing in an emulsion. Different breakdown mechanisms are shown in figure 16 (von Lange and Kurzendorfer, 1974). They are generally described as creaming, flocculation, coalescence, phase inversion and/or phase separation. The breakdown mechanisms have been extensively investigated (Menon and Wasan, 1985). Probably, a combinations of these mechanisms are involved in the destabilization of emulsions (Leman and Kinsella, 1989).

4.4.3.1 Creaming
Creaming refers to gravitational forces acting on phases that differ in density. This leads to separation of the emulsified droplets without changes in their droplet size, and to a densely packed phase, see figure 16 (Leman and Kinsella, 1989).

Stability rating (SR) may be used in order to describe the creaming stability of a protein. Tornberg and Hermansson (1977) made a suggestion how to calculate SR. It is determined on the basis of the percentage change of fat in the aqueous lower phase after creaming of the emulsion formed for 24 hr. The following equation was used:

\[ SR = \frac{F_{\text{test}}}{F_{\text{original}}} \times 100(\%) \]  

(6)

where \( F_{\text{test}} \) is the percentage fat of the bottom 5 ml of the sample and \( F_{\text{original}} \) is the initial fat percentage of the whole sample. It is a popular and rapid test. Centrifugation is usually used in order to accelerate the creaming rate of the dispersed phase.

The creaming phenomenon of whey proteins has been investigated using this technique. Soya bean isolate, sodium caseinate and whey protein concentrate were studied. It was demonstrated that more stable emulsions against creaming may be obtained, up to a certain limit, by increasing the emulsifying intensity and time. The upper limit was also dependent on the apparatus used, type of protein and environmental factors. The proteins studied showed similar behaviour with regard to stability-time and stability-intensity dependence (Tornberg and Hermansson, 1977).
Tornberg (1978c) also used this creaming stability technique as a measure of protein-stabilized emulsions. The proteins studied were soya bean protein isolate, whey protein concentrate and a sodium caseinate. Different types of emulsifying apparatus were used, and although the power and energy input were the same, the creaming stability differed as a function of the emulsifying apparatus used. Increased power and energy input increased creaming stability. Among the proteins studied in this investigation, whey protein concentrate seems to give the best emulsions in terms of creaming stability.

Leman et al (1988) studied ES from the extent of creaming for whole milk protein, β-lg, whey protein isolate and micellar casein. The ES was expressed in terms of the height of a separated cream layer as a percentage of the initial height of the emulsion. It was found that, with increasing energy input and protein concentration, ES increased, β-lg showed best ES under the conditions chosen. The order of ES was β-lg > whey protein isolate > milk protein > micellar casein.

4.4.3.2 Flocculation
Flocculation refers to aggregation of emulsion droplets via interactions of macromolecules, adsorbed on the droplet surface. It does not involve rupture of the interfacial film and, therefore, it does not involve any change in the size of the emulsion droplets. Fat globules move as groups rather than individually. Flocculation usually occurs in heated products and on storage.

Flocculation of whey protein concentrate has been studied with the help of microscopy and compared with the degree of flocculation of casein, blood plasma protein and soya protein isolate. It was concluded that whey protein and blood plasma protein as emulsion stabilizers give rise to emulsions with a high degree of flocculation (Tornberg and Ediriweera, 1988).

Bridging flocculation is a destabilizing phenomenon in emulsions which may occur if the amount of proteinaceous emulsifier available during emulsification is too low to cover the whole interface. It involves sharing the protein aggregate between two droplets of oil in an emulsion, thus utilizing the protein more efficiently (Dickinson et al, 1989b).

Bridging flocculation in binary protein-stabilized emulsions has been observed with the help of light microscopy (Dickinson et al, 1989b). The systems studied were gelatin-caseinate and gelatin-whey protein. It was concluded that the quantity and composition of the emulsifier determined the state of droplet aggregation. Probable mechanisms involved are discussed in the article, and it is also pointed out that more
investigations are needed in order to elucidate the mechanisms involved.

The mechanisms discussed so far, i.e. creaming and flocculation, will affect the emulsion behaviour and its physical properties, but they will leave the particle size distribution unchanged and the flocculated droplets may be readily dispersed again as a result of the weak nature of the interactions.

4.4.3.3 Coalescence
Coalescence denotes the accretion of droplets. This involves rupture of the interfacial film, joining of globules and a reduction in interfacial area, i.e. a mechanism that will gradually result in a separation of the emulsion into an oil phase and an aqueous phase. Coalescence is the most serious form of emulsion destabilization. It is usually preceded by flocculation, creaming, sedimentation and/or Brownian movement as contact between the globules must precede (Nawar, 1985). As the droplets merge, a thin film of the continuous water phase is trapped between the drops. This thin film determines the degree of stability of the emulsion, and the rate of thinning of this film determines the time required before the two drops make contact. When the film is critically thin, it will break and this will result in unition and coalescence of the droplets unless the interfacial film is very stable. Thus, the coalescence mechanism is divided into two processes: film thinning and film rupture (Brown, 1968). Coalescence occurs frequently at the time of formation of the emulsion (recoalescence) and also when the emulsion is subjected to stresses, such as freeze-thaw cycles or drying (Tornberg and Ediriweera, 1988).

Because of the relatively high stability of protein-stabilized emulsions, it is relatively difficult to measure coalescence instability. The main method to follow coalescence is to follow the change in droplet size as a function of time or process to be studied. This may be done with, for example, the help of microscopy techniques (Mita et al., 1973;1974), spectroturbidimetric methods, photomicrography, Coulter counting, etc (Walstra, 1968). An accelerating technique for measuring coalescence stability of protein-stabilized emulsions has been developed. It involves extracting the oil coalesced with hexane. The coalescence stability of soya protein isolates, caseinate, whey protein concentrate and blood plasma-stabilized emulsions has been estimated with the help of the hexane extraction method. Caseinates were proved to be the best stabilisers, the whey protein concentrate and the blood plasma showed medium stabilising, power whereas the soya proteins gave rise to emulsions of minimal stability (Tornberg and Ediriweera, 1988).

The coalescence properties of a commercially available whey protein isolate has been studied when modified by progressive oxidative sulfitolysis in order to cleave
disulphide bonds. It was proved, with microscopy techniques, that as the extent of
sulfitolysis increased, the coalescence decreased (Klemaszewski and Kinsella, 1991).

4.4.3.4 Phase inversion/separation
Phase inversion and phase separation are two destabilizing mechanisms existing in
emulsions. Phase inversion refers to a change from an oil-in-water emulsion to an
water-in-oil emulsion. Phase separation refers to a change in the emulsion which
leads to two separate liquid layers.
4.5 Factors affecting emulsion stability

ES depends on many different factors, for example, the emulsifying agent, droplet size, net charge, ionic strength, viscosity of the continuous phase, density of the droplets, gravitational field, temperature, structure of the emulsifying agent, mechanical and physical properties of the adsorbed film, the emulsifying process, existence of liquid crystals, etc (Kinsella, 1984; Leman and Kinsella, 1989; Tornberg and Hermansson, 1977). Thus, ES depends on the factors involved in the emulsion and its existence and, of course, an understanding of how the emulsion system works and behaves under different environmental conditions would improve the ability to increase ES. Some of the factors mentioned are more important than others regarding ES, and they have been more or less extensively investigated. Some of the important factors are reviewed below.

4.5.1 Droplet size

The droplet size is supposed to have a strong influence on the stability of the emulsion. Larger drops usually result in more rapid destabilizing mechanisms, for example, creaming and coalescence. Droplet size is mainly governed by the type of equipment used and on the energy intensity applied (Leman and Kinsella, 1989; Tornberg, 1990; Tornberg and Ediriweera, 1988). Figure 17 shows a decrease in droplet size, i.e. increase in fat surface area, with net power consumption of whey protein samples. Higher energy intensity gives smaller droplets.

In order to study ES-effects and improve upon them, it is important to quantify and to more exactly describe the emulsification process, i.e. to more or less standardize it. This will make it possible to relate different investigations and results to each other. When emulsification is standardised, it may also be possible to relate larger droplets to recoalescence (Tornberg and Lund, 1978).

Determination of the size distribution of the droplets in an emulsion is far from easy. The size varies considerably and many complications arise from this. Some of the different methods existing for this determination are discussed by Walstra et al (1969). They include ordinary and fluorescence microscopy, photomicrography, Coulter counting and spectroturbidimetry. All the methods have advantages and disadvantages and the applicability will depend on the problem studied. It is, however, advisable to use more than one technique in order to obtain reliable and reproducible results.
Fig. 17 Fat surface area of protein-stabilized emulsions as a function of net power consumption after emulsification with a valve homogenizer using 10 passes through the recirculating system. Symbols: (a) ●—●, soy protein (0.2-7); ▲—▲, whey protein concentrate (0.2-7); ■—■ caseinate (0.2-7); (b) ○—○, soy protein (0-7); □—□, whey protein concentrate, (0-7); □—□, caseinate (0-7). (0.2-7) indicates that the aqueous solution contains 0.2M NaCl and the pH is 7, (0-7) indicates an aqueous solution at pH 7. (Tornberg, 1978b).

A method for estimating the globule-size distribution with the help of turbidity measurements on diluted emulsions has been proposed by Walstra (1968). A specific turbidity spectrum was then calculated from the optical density of the emulsions at several wavelengths, the concentration and from refractive indices. This was compared to theoretical spectra, computed for various globule-size distributions. The
results obtained seem to agree well with those of other methods, for example, Coulter counting and microscopy. Further advantages with the technique is that reproducible results are obtained and it is easy and rapid to perform. One disadvantage is the elaborate preliminary calculations. Walstra also points out that the extreme dilution of the emulsion, which freezes the size distribution, and the high reproducibility of the results obtained make the method very useful for studying coagulation.

4.5.2 The nature of the interfacial film

ES is dependent on the nature and on the mechanical and rheological properties of the interfacial film. Proteins usually adsorb irreversibly and the properties of the film i.e. thickness, cohesiveness, charge, etc, depend on the proteins used and on the conditions of emulsification. The amount and type of protein, i.e. molecular weight, conformation, and also the conditions in the aqueous phase, such as pH, ionic strength, which may affect protein conformation and aggregation, determine the surface excess of the protein (Kinsella, 1984; Leman and Kinsella, 1989).

Surface viscosity is an important indicator of film stability. It reflects internal friction and resistance to flow and is a measure of the mechanical strength and the extent of protein-protein interactions in a film (Leman and Kinsella, 1989). The surface viscosity may be measured by studying the shear properties of the protein films at interfaces. This is done by monitoring the displacement of a glass disc suspended in the interface and obtaining creep compliance curves. The response of the film is modelled by elastic springs and dashpots, ordered in parallel and in series (Graham and Phillips, 1976; Phillips, 1981). Proteins with random coil-type properties form films with little cohesiveness, in contrast to globular proteins, for example β-lg, that conceivably retain a major portion of their tertiary structure and therefore form viscous films with good mechanical properties that may form more stable films in emulsions (Kinsella, 1984). The viscoelastic properties of BSA-films have been investigated and have proved to be maximal at the isoelectric point (Phillips, 1981). This is consistent with the knowledge that the viscosity of surface films is at a maximum in films where the greatest degree of protein-protein interaction occurs.

Protein load is involved in ES. It is generally known that a high protein concentration in the bulk phase results in a higher protein load than a low protein concentration. See figure 18. If there is a low protein concentration in the bulk phase, it is also more probable that the protein will unfold more at the interface (Walstra, 1988; Tomberg and Ediriweera, 1988).
The protein load for whey protein concentrate, soya protein isolate and sodium caseinate has been studied using the method based on separation of two dispersed phases and subsequently measuring the protein content in one of the phases or in both. The protein load proved to be high for whey proteins at small surface areas and, as whey proteins are small molecular complexes, this indicates that a multimolecular layer is formed. This multilayer formation is explained by the phenomenon that unfolding of whey protein molecules at the interface induces exposure of hydrophobic groups, leading to further adsorption of protein from the bulk thereby enhancing multilayer formation (Tornberg, 1978b).

![Graph](image)

**Fig.18** Average surface load ($\Gamma$) as a function of the concentration of protein (C) per unit oil surface area (A) created by emulsification. The dotted line corresponds to complete adsorption of all protein. The results may vary depending on conditions like pH and ionic strength (Walstra, 1988).

The protein film may involve electrostatic and steric forces, i.e. charge density, bulky side chains or substituent groups, hydrated groups projecting into the continuous aqueous phase, etc, which may affect the ES greatly. A high charge density on the globule film results in an electrical double layer around the dispersed globule. This
induces repulsive forces and counteracts attractive van der Waals forces. A proper balance between these two forces results in an increase in ES. The steric factors inhibit the globules from merging with each other and, consequently, also prevents attractive van der Waals and hydrophobic forces from becoming effective (Leman and Kinsella, 1989).

4.5.3 Liquid crystals

Friberg and coworkers observed liquid crystal regions in emulsions which consisted of several mesomorphic phases in equilibrium with each other and with the isotropic solution (Friberg et al, 1969). In 1971 the stabilizing effect of liquid crystalline phases formed at the surface of oil droplets in o/w emulsions was demonstrated (Friberg and Rydhag, 1971).

Weak interactions between the emulsifier, oil and water result in the formation of a liquid-crystalline multilayer around the droplets. This generates a barrier which causes a reduction in van der Waals forces and an improvement in ES. The liquid crystals possess a high viscosity compared to that of water, indicating the importance of these structures for ES (Nawar, 1985; Krog et al, 1989).

Liquid, crystalline lipid-water phases are formed by many naturally occurring polar lipids or industrially produced emulsifiers and surfactants. These phases form a lamellar structure at the interface and provide stability against coalescence. The lamellar structure is preferably formed in emulsions containing a lipophilic emulsifier in the oil phase and a hydrophilic emulsifier in the water phase. A combination of these two emulsifiers, in an optimal ratio, provide stability of the lamellar phase (Krog et al, 1989).

4.5.4 The continuous medium

A high viscosity of the continuous medium provides ES. The rate and number of collisions are principally determined by the viscosity of the continuous medium and are minimized as it increases (Leman and Kinsella, 1989; Nawar, 1985). Thus, higher viscosity causes a significant delay in flocculation and coalescence (Nawar, 1985).
4.5.5 Miscellaneous factors

Many miscellaneous factors may affect ES in a different way. Some of them will be discussed briefly here.

In a previous section, the solubility of proteins has been correlated with emulsifying properties. The relation between ES and solubility is that a higher concentration of soluble proteins facilitates the adsorption and formation of thicker interfacial films. It also encourages smaller droplets, which are more conductive to ES. This may also be seen in figure 18, where the protein load as a function of protein concentration is depicted.

A temperature increase generally results in more disordered systems, i.e. higher entropy of the system. This tends to accelerate all breakdown mechanisms in emulsions. In some cases, gelation may be induced by a temperature increase, and this gives rise to an viscosity increase, leading to an increase in ES, since the mobility of the globules will be reduced (Halling, 1981).

If small amphiphilic molecules are added to an emulsion, they may stabilize emulsions better by displacing the surfactant protein film (Halling, 1981).
4.6 Classifying emulsifiers

Several methods have been introduced in order to choose an appropriate emulsifier for an emulsion system. Emulsifiers have been classified according to whether they are anionic, cationic or nonionic, naturally occurring or synthetic, function as surface-active agents, viscosity enhancers, solid absorbents or whether their hydrophobic or hydrophilic properties are most prominent (Nawar, 1985). As mentioned before, proteins are usually used in food emulsions (Tornberg, 1990). Two common ways to classify an emulsifier will be discussed here, the hydrophilic-lipophilic balance (HLB) concept and the phase inversion temperature (PIT).

4.6.1 HLB-system

The hydrophilic-lipophilic balance system is based on the fact that emulsifiers are amphilic compounds, i.e. they contain both hydrophobic and hydrophilic groups, and are thus soluble in both phases. The emulsifier is generally more soluble in the continuous phase, and the type of emulsion formed, i.e. o/w or w/o, may be predicted on the basis of the relative hydrophilic-lipophilic properties of the emulsifiers (Griffin, 1949). According to the HLB-concept, each surface-active agent can be assigned a numerical value representing its hydrophilic-lipophilic balance. One can say, as a rule, that HLB-values in the range of 3-6 promote w/o emulsion and values between 8 and 18 promote o/w emulsions (Nawar, 1985).

It has also been suggested that the HLB-values are algebraically additive. The HLB-value of a blend of two or more emulsifiers may then be obtained by simple calculation, and the blend of emulsifiers which produces maximum ES can be easily obtained (Nawar, 1985). However, it has been proved that this is not always the case.

The HLB-concept is very useful when comparing emulsion-forming or stabilizing properties, but it suffers from a number of limitations, for example, commercial emulsifiers usually consist of a group of compounds rather than a single component. This makes direct calculation based on chemical properties, such as the hydrophilic-lipophilic balance, difficult. Another drawback is that the HLB method does not take into consideration such factors as emulsifier concentration, mesomorphic behaviour, temperature, ionization of the emulsifier, interaction with other compounds present, or properties and relative concentrations of the oil and aqueous phases, etc (Nawar, 1985).
4.6.2 PIT-phase inversion temperature

PIT is also a concept used in order to select a proper emulsifier. It relates directly to the temperature, as this is an important factor affecting the emulsion-forming characteristics of a surface-active agent. An emulsifier that is soluble in water at a certain temperature may be preferentially soluble in oil at a higher temperature, at which hydrophobic interactions become stronger. Determination of this phase inversion temperature provides a useful basis for emulsifier selection. A strong positive correlation has been observed between the PIT of emulsifiers and ES (Nawar, 1985).
5. GELS

Through the years there have been several attempts to define a gel. It is not an easy task and no one has been able to come up with a definition that holds generally. One reason may be that the various gels existing have different structures and are studied by scientists with different backgrounds, e.g. chemists, physicists, engineers, biologists, medical researchers etc. However, there is a need today to produce a clear definition because this would probably increase communication between scientists in different areas and increase the knowledge. A short review of the existing definitions of gels will be given here.

A well-known definition was given by Hermans (1949). He defined a gel as a coherent, colloid, dispersed system exhibiting mechanical properties characteristic of a solid state. The gel should consist of at least two components, dispersed component(s) and dispersion medium, extending themselves continuously throughout the whole system. This definition excludes one-component systems and is consequently in conflict with the Flory-Stockmayer gelation theory. See section 5.1.1 for details of this theory.

Several years later Flory (1974) proposed a classification of gels based on structural criteria:

a. Well-ordered lamellar structures, including gel mesophases.

b. Covalent polymeric networks; completely disordered.

c. Polymer networks formed through physical aggregation: predominantly disordered, but with regions of local order.

d. Particulate, disordered structures.

A phenomenological definition was proposed by Burchard and Ross-Murphy (1988) not so many years ago, stating that:

"...gels all possess at least one property which can stand as the operational definition of a gel; they possess a plateau in the real part of the complex modulus extending over an appreciable window of frequencies - i.e. they are, or can be coaxed under appropriate conditions to be, viscoelastic solids."

Almdal et al (1992) qualified this phenomenological definition made by Burchard and Ross-Murphy and proposed the following definition:

a. A gel is a soft, solid or solid-like material of two or more components one of which is a liquid, present in substantial quantity.

b. Solid-like gels are characterized by the absence of an equilibrium modulus.
by a storage modulus, $G'(\omega)$, which exhibits a pronounced plateau extending to times at least of the order of seconds, and by a loss modulus, $G''(\omega)$, which is considerably smaller than the storage modulus in the plateau region.

The ability to form a gel is an important technological property which is utilized in different food products. Lately, the research on whey protein gels has been concentrated on their use as fat substitutes in different food products with the idea of lowering the fat content. This is possible because the viscoelastic properties and surface activity, discussed in the chapter for emulsions, of the gels under suitable conditions, symbolize fat very well. This trend is clearly noticed when reading the patent section in this review.

This chapter will deal with whey protein gels, both as a whole and individually, their formation, existing forces, microstructure, and rheological properties under various environmental conditions.
5.1 Gelation

In order to create a gel, the protein conformation has to be altered in some way, for example, by heat treatment, changes in pH, action of enzymes, etc (Mangino, 1992). This will make new interactions possible and, if protein-protein and protein-solvent interactions lead to the formation of a three-dimensional network capable of entraining water molecules, a gel is likely to form. The gel will behave as a solid material, and the water associated with the gel network may be envisaged as "pockets" of bulk-phase water. The entrapped water will freeze at normal temperatures, and it will be freely available to participate in chemical reactions (Mangino, 1984). The most common way to induce gelation of whey proteins is by heat treatment, and the chief emphasis in this chapter will be on this technique.

5.1.1 Gelation mechanisms

Gelation theories and phenomena have been reviewed by Stading (1991). Some of the classical theories widely used in order to explain gelation were originally developed by Flory and Stockmayer (Flory, 1941; Stockmayer, 1943).

Fig.19 Formation of a gel by intermolecular linking of a 3-functional species. The angles are not meant to represent real bonds (Ross-Murphy, 1984).
In these theories, the molecules are represented as graphs with reactive ends only. A minimum of three reactive ends is required in order to form a three-dimensional network. Otherwise, only strands, not able to constitute a network, are formed. In figure 19 a hypothetical "unit" with three reactive ends is illustrated. These units interact by two hooks forming a cross-link. If there are enough molecules and reactions, a three-dimensional network may form.

The concentration of molecules is an important factor. There even exists a well defined critical concentration, C₀, below which no continuous network is formed. Above this concentration the gel phase and the storage modulus will increase (Ross-Murphy, 1984). This critical point is usually denoted the gel point and will be discussed later in section 5.1.3.

A widely used gelation mechanism was proposed by Ferry (1948) consisting of two steps. The following reaction was proposed:

$$\text{xP}_N \rightarrow \text{xP}_D \rightarrow \text{(P}_D)_{\text{x}}$$

(7)

$P_N$ denotes the native protein, $P_D$ the denatured protein, x the number of molecules and $(P_D)_x$ the network formed. He proposed that the final gel state corresponds to aggregates of partly denatured protein molecules. The first step, the denaturation step, is usually induced by heat and is reversible (see section 2.1). The second step, indicating the network formation, may also be induced by heat and/or by cooling. This step is irreversible, but a reversible gel state has been postulated in some cases for whey protein gels, see section 5.2.5 (Rector et al, 1989; Beveridge et al, 1984). If the second step is reversible, the intermediate state is called a progel state (Hermansson, 1979).

Network formation by aggregation before denaturation has also been suggested (Hermansson, 1978). This can be described as

$$\text{xP}_N \rightarrow \text{N}_x \rightarrow \text{(P}_D)_{\text{x}}$$

(8)

This behaviour has been noted by Hermansson (1979) for whey protein solutions. She concluded that, at intermediate pH-values, the turbidity started to increase at temperatures lower than the denaturation temperature. Stading and Hermansson (1990) also noted a similar behaviour for whey protein when studying the gelation temperature at different pH-values and comparing it to the denaturation temperature. This is also discussed in section 2.2.4.

Recently, a percolation model has been proposed by Kallala et al (1992). It suggest that the selection of the growth process and of the final state may result from the
relative reactivities of monomers with different chemical environments. The model allows the system to switch during its polymerization from linear growth to isotropic branching, and from isotropic recombination of branched polymers to dense growth through capture of monomers. The formed gel may have a fractal dimension ($D_f$), ranging from $D_f<2$ (gelation of branched polymers) to $D_f=3$ (precipitation of dense particles) depending on the value of the parameter describing the chemical reactivity.

5.1.2 Kinetics of gelation

A gel network is characterized by a certain degree of order, which can be obtained if the second step in the, by Ferry, proposed gelation mechanism above is slower than the first one. This will give the denatured molecules time to arrange themselves and interact at specific points, thus forming a three-dimensional network capable of immobilizing a large amount of water. The resulting network, formed when the second step is slower, can be expected to show lower opacity and higher elasticity than if step one and step two occur simultaneously, or if step two occurs before step one (Hermansson, 1979).

The kinetics of gel formation are sensitive and highly dependent on the environmental factors that determine the behaviour of proteins. It may be possible to control the kinetics to some extent, by choosing suitable environmental conditions which favour the preferred network. For example, as pointed out in the chapter on denaturation, the environmental conditions favouring denaturation do not favour aggregation. Some factors involved in the kinetics will be discussed briefly below.

The selection of the processing temperature is sensitive as it will balance the rate of protein unfolding with that of aggregation. If the heating conditions are extreme, step two may be faster, or as fast as, step one, and the gel structure formed will be adversely affected. Then the protein molecules will not have time to align themselves in an ordered network, and the result will be poorly hydrated aggregates or precipitates, not able to hold water (Mulvihill and Kinsella, 1987; Hermansson, 1979; Mangino, 1984).

The pH-value and the salt concentration are dependent factors of the kinetics. The energy barrier for aggregation is maximal at extreme pH-values and low ionic strength, because under such conditions the protein unfolds and its ionic nature is revealed. As the salt concentration is low, no shielding of charged areas on the protein by ions will take place. The charged areas will induce repulsion in the protein chains, preventing gel formation (Mangino, 1984). Thus, the activation energy of
denaturation is minimized under these conditions, i.e. extreme pH-values and low salt concentrations, and the aggregation may be suppressed prior to the denaturation step.

In contrast, at the isoelectric pH the net charge of the protein is zero. It is around pH 5 for whey protein, pH 5.2 for β-lg. At this pH, aggregation is likely to occur, as no electrostatic repulsion exists between the molecules. The activation energy of denaturation is maximized at this pH-value, and the denaturation step may be suppressed prior to the aggregation step, resulting in aggregates instead of a gel. A proper pH adjustment may be necessary to achieve a proper balance between the rate of denaturation and aggregation, as well as between forces of attraction and repulsion, discussed later, between adjacent protein chains, which are necessary to achieve a protein gel (Mangino, 1984).

5.1.3 Gel point

As mentioned before, in order to form a gel, a minimum protein concentration is required for network formation, which expands the whole volume. This is called the gel point. At too low a protein concentration, a network which expands the whole volume is not able to form, and the result is a precipitate. As the protein concentration is increased, a gel network may be formed throughout the whole volume. When the protein concentration is increased above the gel point, the gelling time is reduced and the gel strength increased (Mulvihill and Kinsella, 1987). The gel point is, of course, dependent on environmental factors such as pH, salt concentration, heating rate, etc. as the forces and bonds constituting gel networks are dependent on these factors.

The gel point is a phenomenon that has received considerable attention, and it is measured in several different ways. For example, dynamic rheological measurements can be used to follow the network formation and the gel point can be defined, rheologically, as the point where the storage modulus, $G'$, starts to increase. $G'$ is the elastic part of the complex modulus $G^*$. Another definition of the gel point that is used is that it is the point where the phase angle equals 45°, independent of frequency. This is the point where $G'$ and $G''$ perform a crossover. $G''$ is the loss modulus, describing the viscous part of $G^*$. Confusion arises since this definition of the gel point, i.e. were $G'$ and $G''$ perform a crossover, is suitable for some network polymers and not for others.
Winter (1987) has modelled the rheological behaviour at the gel point and discussed the validity of the $G', G''$ cross-over definition. Stress relaxation at the gel point was found to follow

$$G(t) = St^{-n}$$  \hspace{1cm} (9)$$

where $S$ is the strength, which depends upon the flexibility of molecular chains and cross-links and on the cross-link density at the gel point. $n$ is the relaxation exponent and varies between 0 and 1. Stress relaxation measurements are performed by measuring the stress during an applied constant strain. At $n=0.5$, the relaxation modulus, $G(t) = St^{0.5}$, gives corresponding moduli:

$$G'(T, \omega) = G''(T, \omega) = \sqrt{\pi/2S(T)\omega^{1/2}}$$  \hspace{1cm} (10)$$

and the loss tangent:

$$\tan \delta = G''/G' = 1$$  \hspace{1cm} (11)$$

i.e. $G'$ has the same value as $G''$ for any frequency and temperature at the gel point. When $n$ equals 0.5. This is the only time when the gel point can be defined as the cross-over point. This behaviour has been observed for stoichiometrically balanced network polymers. When $n$ is not equal to 0.5, detection of the gel point becomes more complicated. The dynamic moduli of the system do not coincide at the gel point, rather they are parallel and obey the following reaction:

$$G' = \frac{G''}{\tan(n\pi/2)} = \frac{\pi}{2\Gamma(n)\sin(n\pi/2)}S\omega^n$$ \hspace{1cm} (12)$$

Thus, the gel point has to be found through the power law of relaxation behaviour. For $n=0.5$, the gel point occurs earlier than the crossover. This behaviour has been found for stoichiometrically imbalanced systems. This parallel behaviour is rather hard to measure for biopolymer gels, since the measuring signals are very small and a lot of noise exists near the gel point. However, the parallel behaviour has been observed on polyvinyl chloride (PVC)/di-2-ethylhexyl phthalate (DOP) systems, and it will be depicted in figure 20, where log $G'$ and log $G''$ are plotted against log $\omega$ for the ageing time of the crosspoint. The curves have a slope of 0.77, corresponding to $n=0.79$. It is impossible to determine if this system is in its critical gel state by dissolving it in a suitable solvent. Thus, unfortunately, its validity in accordance with the gel point definition made by Winter, described above, is not possible to check, but rather a good parallel behaviour may be depicted in figure 20 (te Nijenhuis, 1988).
Fig. 20 Double logarithmic plot of the dynamic moduli of 10% (w/w) PVC/DOP against angular frequency. Ageing time 0.75 h; ageing temperature 90°C (te Nijenhuis, 1988).

A third definition of the gel-point is that it is the point where $G''$ has its maximum (Stading and Hermansson, 1990). Definitions of the rheological terms used above are reviewed in section 5.4.1.

Stading and Hermansson (1990) used all three definitions when studying the gel point of β-lg gels. It was concluded that the gelation temperature calculated from the phase angle at 45° and as $G'$ starts to increase did not differ from each other, whereas the gelation temperature, measured as $G''_{max}$ differed substantially. They studied the gel point as a function of pH, temperature and heating rate.

In figure 21 the critical gel concentration for a β-lg-solution is plotted against pH. It is measured with the help of dynamic measurements as the point where $G'$ > noise. At high and low pH-values, the critical concentration is quite high compared to that at intermediate pH-values. This reflects the difference in the micro-structure of the gels. It implies that the network at intermediate pH-values is much more open than that at low and high pH-values.
Fig. 2.1 The critical β-lactoglobulin concentration required to form a gel as a function of pH (Stading and Hermansson, 1990).

A gel point definition of weak gels, used in the literature, is where $G' \geq G''$ in a broad frequency range and $G' \neq 0$. Tangens delta should also be independent of frequency. This is a simple definition and easy to perform on biopolymer gels (Hermansson, personal discussion).
5.2 Forces in a gel

5.2.1 Theory

The ability of denatured proteins to form new bonds and interactions leading to a gel network depends upon the protein and its amino acid composition, molecular weight, net hydrophobicity, concentration and free sulphhydryl content (Shimada and Matsushita, 1980b) and on the environmental conditions, such as heating and cooling rate, temperature, pH, salt concentration, calcium concentration, etc (Mangino, 1984). In principle, any factor that changes the conformation of the protein, and by that its effective charge and hydrophobicity/hydrophilicity, will induce new bonds and forces governing the formation of a gel network under suitable conditions.

The balance between forces underlying chain-chain and chain-solvent interactions determines the mechanism involved in gel formation. Chain-chain forces may result in mechanisms such as precipitation, association, aggregation, flocculation, as well as coagulation. They may also affect the conformation of the protein, i.e. the helix content, native structure and three-dimensional structure. The mechanisms determined by chain-solvent forces are solubilization, dissociation, swelling and denaturation. The conformation encouraged by these forces is the coil (Hermansson, 1979).

If a gel is to be formed, a balance between attractive forces necessary to form a network and repulsive forces necessary to prevent its collapse is required. If the network is too weak, i.e. too many solvent-protein interactions, fluid flow will be possible and a true gel will not form. On the other hand, if the network is too strong, i.e. too many protein-protein interactions, it will collapse and water will be expelled from the structure (Mangino, 1992).

As already mentioned, the formation of a gel usually starts with denaturation. The denaturation of whey protein has already been discussed in chapter 2. and it is a common fact that it involves unfolding of the protein molecule, reflecting an increase in chain-solvent interactions. As this proceeds, the conformation of the protein is altered, its native bonds and interactions are thereby broken and hydrophobic areas, previously hidden in the interior of the molecule, are free and able to take part in new interactions. This encourage the amount of water, tightly bound to the protein, to increase (Mangino, 1984, 1992). Under suitable conditions, new bonds are then able to form and a three-dimensional network may be established.
This discussion leads to the conclusion that the forces involved in gel formation are those involved in the native structure of the protein (Clark et al., 1981). Thus, for whey protein, electrostatic and ionic interactions (such as calcium bridges and other divalent ions) (Mulvihill and Kinsella, 1988), hydrogen bonding (enhanced at low temperatures), hydrophobic interactions (enhanced at high temperatures) (Voutsinas et al., 1983; Kohnhorst and Mangino, 1985) and disulphide crosslinks (Watanabe and Klostermeyer, 1976; Mangino, 1984) are known to represent the attractive forces, whereas electrostatic repulsions and protein-water interactions represent the repulsive forces (Cheftel et al., 1985; Clark and Lee-Tuffnell, 1986).

The forces involved in whey protein gelation have been the subject of many investigations and the extent and nature of their contribution to the gel network under different environmental conditions have not yet been elucidated. Many hypotheses exist in order to explain the gel network and the large water-retention capacity. One hypothesis suggests that, after denaturation, unmasked CO and NH groups of the protein, negatively and positively polarized respectively, create an extensive system of water multilayers. Upon cooling, the protein molecules may then interact, through hydrogen bonds, to provide the structure necessary to immobilize the free water. Another possible mechanism is that pores in the protein network hold water through capillary forces (Cheftel et al., 1985).

A probable and important effect on gel formation may come from van der Waals forces. These forces are always present between atoms and molecules and, because of this, they play a role in a host of important phenomena, for example they are present in maintaining the native structure of the protein and are, as stated above, involved in the formation and maintenance of the gel. the strength of solids, the flocculation or aggregation of particles in aqueous solutions and the structure of condensed macromolecules such as proteins and polymers, etc (Israelachvili, 1985). The forces are small, and the nature of the interaction is related to the distance between the atoms and/or molecules. At a distance greater than 10 nm, they develop an attractive force, and as the distance is lessened, a repulsive force will develop. The forces also tend to align or orient the molecules, though this effect is usually weak. The van der Waals forces perform nonadditivity, i.e. the interaction between two bodies is affected by the presence of other bodies (Israelachvili, 1985; Cheftel et al., 1985).

In one investigation, the effect of temperature on the viscoelastic properties of whey protein gels was studied. The conclusions were that "while covalent cross-links play a role in whey protein gel network structure, noncovalent cross-links predominate in maintaining the structure" (Katsuta and Kinsella, 1990). A similar study dealt with
the effect of protein concentration on the viscoelastic properties of whey protein gels. It was concluded that non-covalent forces contribute proportionally more to the elastic modulus of whey protein gels at lower protein concentration (Katsuta et al, 1990). These investigations were performed with the help of creep experiments, which are performed during an applied constant stress; see Stading (1991) about creep measurements.

One reason for the many results and hypotheses about the force or forces contributing to certain structures and properties may be that cooperativity exists to a greater extent than is generally recognised. Another reason is that the weak forces may contribute more to the network structure than is so far believed.

5.2.2 Covalent forces

The covalent forces, i.e. disulphide bonds, involved in the gel formation of whey proteins have been investigated, and it has been concluded that there is an optimal sulphydryl concentration required for gel formation. It has also been concluded that the rate of gelling depends on the SH-content. This was proved by heating a test tube with whey protein solution. When no flow was possible in the tube the gelation was assumed to be finished (Hillier et al, 1980). The covalent crosslinking may be obtained by SH oxidation into S-S bonds and/or by SH-induced S-S interchange reactions (Shimada and Cheftel, 1989).

β-lg is the largest contributor of free SH to whey (Mangino et al, 1987). A good correlation between the sulphydryl content of whey protein concentrate and its content of β-lg has been demonstrated (Kim et al, 1987). It has been suggested that the concentration of native β-lg should be related to gel strength, since disulphide bonds govern elasticity and gel strength (Shimada and Cheftel, 1988; Mangino, 1992).

The SH-activity is quite pH-dependent. At low pH-values, the SH groups are relatively inert and do not contribute to the gel structure; instead, hydrogen bonds have been suggested to be responsible for the network of gels (Shimada and Cheftel, 1988, 1989). This is true of pH-values up to 7.75. At higher pH, the SH-content of β-lg becomes highly reactive, and this catalyzes sulphydryl-disulphide interchange reactions leading to covalent cross-links (Mangino et al, 1987). Therefore, at higher pH-values, there is a good correlation between SH-content and gel strength (Mangino, 1992; McKenzie, 1971).
5.2.3 Hydrophobic interactions

Hydrophobic interactions are strong attractions existing between nonpolar molecules or parts of molecules and surfaces in water (Israelachvili, 1985). Usually, a denaturation of molecules is needed to reveal their hydrophobic parts, leading to strong attractions governing network formation. Regarding their contribution to whey protein gel formation and strength, one can conclude that an optimal level is required, beyond which gel strength is weakened. It should be noted that the effective hydrophobicity of proteins depends on both processing conditions and on the environmental conditions under which the measurements are made, since the degree of unfolding of the molecule and, thereby, the hydrophobicity are determined by these factors (Mangino, 1992). In commercial whey protein concentrates, this optimal level is not reached and one can see a positive correlation between gel strength and protein hydrophobicity, measured by alkane binding (Kohnhorst and Mangino, 1985). Voutsinas et al (1983) also found a positive correlation between the hydrophobicity of unfolded whey proteins, i.e. the effective hydrophobicity, and gelation. This was measured fluorometrically.

5.2.4 Electrostatic and ionic interactions

The ionic nature of the protein is revealed during denaturation, and new electrostatic and ionic interactions governing gel formation are able to take place. As the protein chains contain charged groups, repulsion and/or attraction between them exists. At low and high pH-values, these charges are important, and the repulsion between the protein chains is large, inhibiting gel formation. These charges may be reduced by addition of salt, because of the attachment of ions at the charged areas and this will weaken the repulsive forces and facilitate gel formation. However, to high a salt concentration may induce excessive protein-protein interactions, thereby causing the network to collapse. Obviously, there exists an optimal salt concentration for obtaining the best balance between protein-protein and protein-solvent attractions, inducing gelation. Many studies have been performed on the effect of salt on gel formation (Schmidt et al, 1979; Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991a,b; Mulvihill et al, 1990). The effect of salt addition on denaturation and aggregation has been discussed, see sections 2.6.2 and 3.2.2, and later in this section, its effect on the viscoelastic properties of whey protein gels will be studied.

The formation of whey protein gels is largely dependent on the availability of Ca²⁺ ions and on their formation of bridges between adjacent, negatively charged protein chains after unfolding. This crosslinking assists the formation of networks. There
have been suggestions about the existence of two calcium binding sites on α-la at neutral pH with different binding capabilities. The first has a high affinity for Ca\(^{2+}\). On high purification of α-la, it still contains a stoichiometric quantity of Ca\(^{2+}\), namely one Ca\(^{2+}\) per α-la molecule. The second sites have a lower affinity for Ca\(^{2+}\) but a further stabilization of the native structure takes place on addition of Ca\(^{2+}\) (Hiraoka et al., 1980). However, it is not easy to distinguish between calcium ions tightly bound and calcium counterions shielding the charge.

An excessively high Ca\(^{2+}\)-concentration induces too many bridges, and the protein matrix will then collapse into large, dense, non-continuous aggregates surrounded by the aqueous medium (Kohnhorst and Mangino, 1985; Mulvihill and Kinsella, 1988). It has been reported that commercially available whey protein concentrates may obtain a Ca\(^{2+}\)-content in the range that inhibits gel formation (Kohnhorst and Mangino, 1985). The effect of Ca\(^{2+}\)-concentration on the viscoelastic properties of whey protein gels is discussed in section 5.5.4.

It can be concluded that there probably is an optimal level of calcium for gel formation, and that this optimal level varies with the system in question. For example, some of the calcium may be bound in a different manner, i.e. the conformation of the protein, the composition of other minerals and non-protein components in the system are of great importance, as they can bind the free calcium in the system and, thus, inhibit its cross-linking capability and, by that, gel formation (Kuhn and Foegeding, 1991a).

5.2.5 Reversibility of whey protein gels

The crosslinks in a gel, with the exception of disulphide bonds, are not permanent and may continuously break and reform. Whey proteins usually form thermoirreversible gels and proposals exist that disulphide bonds may be responsible for this (Shimada and Matsushita, 1980a,b; 1981). A reversible gel state in whey protein gels is formed upon cooling and is explained to be due to hydrogen bonds and ionic interactions, i.e. reversible bonds. This may be described as

\[ \text{solution} \rightarrow \text{gel} \rightarrow \text{gel} \]

where the first step is irreversible and induced by heat and the second step reversible and induced by cooling.
Fig. 22 Reversibility of gelation in egg albumen (8%) and whey protein concentrate (22.8%). Gelation temperature is 85°C and, after the initial 30-min heating, the temperature was cycled stepwise between 85°C and 25°C. Three temperature cycles are shown. Note compression and scale changes on the vertical axis. Arrows indicate points of cooling and subsequent reheating (Beveridge et al. 1984).

Rector et al (1989) studied this reversible gel state of whey protein isolate and β-lg with the help of their viscoelastic properties, determined by dynamic measurements. It was concluded that the formation of an irreversible progel state upon heating at 90°C for 15 minutes, and formation of a reversible gel state upon cooling to 6°C and being allowed to set for 12 h, took place between pH 6.5-8.5. The nature of the irreversible progel state was dependent both upon pH and concentration of the whey protein. It involved crosslinking via thiol-disulphide bonds, detected with the help of electrophoresis studies. Large aggregates of β-lg were formed, which contributed to the formation of a viscous solution. Upon cooling, a reversible gel state was formed by weak crosslinks, probably hydrogen bonds and ionic interactions, since these crosslinks are enhanced at low temperatures (Catsimpoolas and Meyer, 1970).
The reversibility of whey protein concentrate gels has also been studied by Beveridge et al (1984) with the help of dynamic viscoelastic studies. The gels were formed by heating a whey protein solution at 85°C for 30 minutes and subsequently cooling it to 25°C for 10 minutes, reheating it again to 85°C for 10 minutes and then cooling it to 25°C for 10 minutes. This temperature cycle was performed three times in order to demonstrate the reversibility of the gel. They found, in principle, the same behaviour as described above. Irreversible gelation was obtained when heating at 85°C, which further set in a reversible manner upon cooling to 25°C, see figure 22. The irreversible gel state was suggested to depend upon the formation of disulphide bonds and hydrophobic interactions, whereas the reversible cold set phenomenon probably depended on ionic interactions and hydrogen bonds.
5.3 Microstructure of whey protein gels

The network structure of a gel is of great importance, since it governs its properties, i.e. texture, fat- and water holding capacity, elasticity, diffusion properties etc., and appearance of the gel.

Many different techniques exist to elucidate the network structure of a gel. Some techniques examine the details of crosslinks at the molecular level, while others examine network structures at the supramolecular level. Microscopical techniques are valuable for studies of network structures, but there might be complications involved in the preparation techniques. Physical techniques, such as spectroscopy, give indirect proof of structural properties (Stading, 1991).

In figure 23, the techniques concerning studies of molecular, macromolecular and supramolecular structures are compared. It also relates the time scales of interest to the distance scales and points out where techniques overlap (Clark and Ross-Murphy, 1987).

Fig. 23 Explanation of "molecular", "macromolecular" and "supramolecular" distance scales, the techniques used to probe these and the approximate time scales involved (Clark and Ross-Murphy, 1987).

Generally, biopolymer gels can form two types of gels, fine-stranded gels and particulate gels. This is also the case for whey protein, usually referred to with the help of studies on β-lg gels (Langton and Hermansson, 1992; Hermansson, 1988;
Harwalkar and Kalab, 1985b). The transition from one type to the other can take place within a small change in, for example, pH, ionic strength, concentration of other components, heating conditions, etc. The charge on the protein determines the type of gel formed. If the repulsion is large, the energy barrier against random aggregation is large and fine-stranded gels are formed. In the vicinity of the isoelectric pH, the repulsion is small and a network of colloidal particles is formed (Clark and Lee-Tuffnell, 1986; Langton and Hermansson, 1992). The size of the particles in the aggregated network is in the order of μm in diameter, whereas the strands in the fine-stranded network have a diameter in the order of nm (Langton and Hermansson, 1992). This size difference is the reason for the fine-stranded gels being transparent and the aggregated opaque. If the size of the grain is small but larger than the wavelengths of the light involved, i.e. visible light, it will enter each particle and be reflected and refracted several times and emerge. The reflected light will then be observed as white. The range of wavelengths for visible light is 400-800 nm, which is in the same range as the size of the aggregated particles, and this is the reason for the opaque appearance of the aggregated gels. The fine-stranded structure is too small, being in the range of nm and therefore no light will be reflected and refracted. Thus, it will appear as transparent (Hecht, 1987).

5.3.1 Fine-stranded gels

Fine-stranded β-lg and whey protein gels have been studied with different microscopy techniques, for example with light microscopy, scanning electron microscopy and transmission electron microscopy (Langton and Hermansson, 1992). It was concluded that below pH 4 and above pH 6, in the absence of salt, the energy barrier against aggregation was large, resulting in the formation of transparent, fine-stranded gels. see figure 24.

A difference exists between the strands formed at low pH and at high pH. At low pH, the strands are stiff and short, whereas at high pH they are long, flexible and more curled. A different behaviour is also noticed at the shifts, i.e. the fine-stranded/aggregated shifts. At the lower shift, pH 4, a mixture of particulate and fine-stranded structures is found, whereas at the higher shift, pH 6, no mixture of structures is found (Langton and Hermansson, 1992).

Harwalkar and Kalab also studied the gel structure of β-lg, with the help of transmission electron microscopy (Harwalkar and Kalab, 1985b). They found that close to the isoelectric pH, the microstructure was composed of compact aggregates, in agreement with the result discussed above. At lower pH, pH 2.5, they concluded
that the network consisted of protein particles as well as a few chain-like structures linked together. Thus, there seems to be a mixture of the gel structures here.

Fig. 24 Transmission electron microscopy micrographs showing the fine-stranded network structure of β-lactoglobulin gels formed at pH 7.5 (Langton and Hermansson, 1992).

In order to explain the disparity between the results obtained from Harwalkar and Kalab (1985b) and the one obtained from Langton and Hermansson (1992), one has to take into account the ionic strength. The gels studied by Langton and Hermansson contained no salt, whereas those studied by Harwalkar and Kalab had an ionic strength (I/2) of 0.2. At low or high pH-values, the protein will contain some charged areas. These will induce electrostatic repulsions in the gel, which will govern the fine-stranded network structure. As salt is added the charged areas will be masked by ions and the electrostatic forces will be reduced, inducing aggregation and an aggregated network structure. This may be the reason for the mixed gel structure observed at pH 2.5. The lower shift is moved to lower pH-values as salt is added and, probably, the same is true of the upper shift. The movement of the pH-boundary may also be due to other factors. These are discussed below in the section "particulate gels".
Inhomogeneities have been observed in the network of fine-stranded \(\beta\)-lg gels with the help of electron microscopy (Stading et al, in press). Their formation depends upon the heating rate at pH 7.5. If it is slow, inhomogeneities are formed and occur as dense and loose regions in the network. The inhomogeneities also occur at the pH near the shifts in gel structure, i.e. around pH 6 and pH 4 (Langton and Hermansson, 1992; Stading et al, 1992). The same inhomogeneities have been observed for \(\beta\)-lg gels at pH 8 on addition of NaCl (Mulvihill et al, 1990). As the NaCl concentration increased, the network shifted from homogeneous, fine-stranded gels to inhomogeneous and then further to particulate gels, reflecting the decrease in electrostatic repulsion due to the masking of charged regions on the protein by ions.

5.3.2 Particulate gels

Between pH 4 and pH 6, \(\beta\)-lg and whey protein form particulate, white-opaque gels. See figure 25.

Fig.25 Scanning electron microscopy micrographs at low magnification of \(\beta\)-lactoglobulin gels formed at pH 5.5 (Langton and Hermansson, 1992).
The gels consist of spherical aggregates linked together like a zig-zag band forming the strands of the network. The charge of the protein is low in this pH-range, and the protein molecules are prone to aggregate. The size of the aggregates differs with pH. The particulate gels have been observed to form two types of network structures, irregular and regular. The regular networks, formed between pH 6-5 have a uniform distribution of particles and pores with larger particles formed between pH 5 to 5.5 and smaller at pH 5.5 to 6. The irregular network is formed at pH 4.5. It consists of both large and small pores, as well as large and small particles. The small particles seem to fuse together and form larger particles with cauliflower-like appearance (Langton and Hermansson, 1992).

Whey protein gels and β-lg gels show almost the same behaviour. The small difference noticed is that the pH-region of particulate gels is expanded for whey protein. For example, aggregated whey protein gels have even been found at pH 9. The gel concentration was 12% and different heat treatments were used to obtain the gels, and it was concluded, with the help of scanning electron microscopy, that the gel structure became coarser with larger aggregates and pores in the network structure, when the heating was increased from 75°C to 95°C, but there were no signs of fine-stranded gels (Hermansson, 1986).

The different pH-ranges in which the aggregated gels are formed may be explained by the fact that whey protein is composed of different proteins with different isoelectric points, see table 1. 85% of the proteins in whey, i.e. β-lg, α-la and BSA, have their isoelectric points around pH 5 and 11%, the IG, have an isoelectric point around pH 6-8, expanding the particulate region to higher pH values. The whey protein also contains both salts and lactose, which may affect the gel structure in different ways. Another important factor may be the pre-heat treatment performed on the protein solution in order to obtain commercially available whey protein products, for example, whey protein isolate/concentrate. Dry-heat treatment of whey protein and β-lg at 80°C for 7 days has been studied. It was concluded that the treatment caused partial denaturation, detected with the help of DSC and progressive polymerization, studied by gel electrophoresis. These reactions may happen during processing and/or storage of commercial whey products, and the polymerization may induce an aggregated gel structure and be one of the main reason for the fluctuations in the pH-range. The products are usually obtained by spray drying, which may cause a particle temperature above 100°C. The polymerization proved not to involve disulphide crosslinks (Rector et al, 1991). How the gelling behaviour and viscoelastic properties of the gels were affected by this heat treatment will be discussed in section 5.5, and this special investigation will be further reviewed in section 5.5.5.
Hermansson (1986), described above, used a commercial whey protein concentrate which was probably both partially denatured and polymerized to some extent. This may be the reason for the aggregated microstructure found at high pH-values.

Clark et al (1981) studied the microstructure of heat-set protein gels with the help of transmission electron microscopy. Among the proteins studied were BSA. It was concluded that the charge of BSA, which may be changed by pH or salt addition to the solution, has a large effect on the structure of the BSA gels formed. This was concluded from microscopy studies, ranging from salt-free gels to gels with high salt concentration. Their microstructure changed from less granular to high granular gels and subsequently, at high salt concentrations, coagulates were formed. It is pointed out that heat induced networks vary widely in pore size distribution and in the shape and thickness of the strands in the network. This is dependent on the mode of aggregation. A very broad distribution and very irregular networks are formed from essentially random aggregation, and more regular and uniform networks are obtained when the mode of aggregation is more defined. The distinction between random and ordered aggregation is not clear-cut. The difference arise from mechanisms derived from the number of bonding sites available per unfolded molecule, their spatial distribution and their relative bonding strengths under the prevailing environmental conditions. These mechanisms depend, of course, on the degree of unfolding prior to aggregation of the molecule. Attempts to find a correlation between network type and degree of unfolding lead to the suggestion that "a substantial protein unfolding does not inhibit ordering of protein molecules during aggregation; apparently it can even encourage it".

The effect of structuring, Cl−, and destructuring, SCN−, anions on the microstructure of β-lg gels has been investigated (Mulvihill et al, 1990). The investigation was performed at pH 8 and the gels studied contained different salt concentrations. At low NaCl-concentration, 50 or 100 mmol/dm³, small aggregates of protein were linked together by fine strands, forming a gel. Increasing the NaCl-concentration to about 200 mmol/dm³ made the structure more evident. However, at even higher NaCl-concentration, 1 mol/dm³, the microstructure consisted of large aggregates. These gels were weak coagula. On addition of SCN−, the gels also had a particulate nature. The network formed was more evenly distributed in the continuous matrix compared to the NaCl-containing gels. On addition of 1 mol/dm³ SCN−, gels consisting of smaller aggregates, crosslinked to give a continuous network, were formed. The suggested explanation for this difference in gel structure is that a greater unfolding of β-lg takes place in the presence of NaSCN than in the presence of NaCl, making the formation of stronger gels possible. This hypothesis was confirmed with the help of rheological measurements (Mulvihill et al, 1990).
5.3.3 Fractal structure

Bremer et al have developed a model which describes the fractal nature of particulate gels (Bremer et al, 1989, 1990). The model assumes that particle aggregation leads to fractal clusters which form a gel network when they occupy the whole volume. This model has been utilized for β-lg particulate gels and the fractal dimension, Df, has been calculated with the help of the concentration dependence of the elastic modulus, G, described by

\[ G = K \phi^{2-D_f} \]  

(14)

where \( \phi \) is the volume fraction of particles and \( K \) is a constant. The \( D_f \) was found to be around 2.3 for aggregated β-lg gels. For the fine-stranded gels, the fractal dimension was around 2.90-2.95, indicating that the gels do not have a fractal network structure or that the model is not suitable (Stading et al, in preparation).
5.4 Rheology of whey protein gels

Food protein gels are usually described in terms of their rheological properties. Since whey protein is used in different food products, this is one of the reasons for the extensive research regarding their viscoelastic properties and factors affecting these properties. Thus, in order to increase the use of whey protein in food products, a knowledge and an understanding of how these factors affect the gel and its appearance is required. In this section, the viscoelastic properties of whey protein gels will be reviewed after a short introduction to the world of rheology.

5.4.1 Basic rheological definitions

Rheology is the science of deformation and flow of matter (Shoemaker et al, 1992). Rheologists study the behaviour of viscoelastic materials and this enables them to characterize the consistency and structure of the material. In rheological tests, the material of interest is deformed and the force measured, or a force is applied to the material and the deformation is measured. Rheological definitions have been summarized and discussed (Whorlow, 1980; Stading, 1991). In this section only the most basic definitions will be reviewed. The material discussed is mainly taken from Whorlow (1980) and Stading (1991).

The measured force, dependent upon the area it acts over, is called stress and defined as

\[ \sigma = \frac{F}{A} \]  \hspace{1cm} (15)

where \( F \) is the force and \( A \) the area.

The deformation is usually denoted by strain, and many different definitions exist, among the Cauchy strain is the most common one, defined as

\[ \varepsilon = \frac{\Delta l}{l_0} = |\lambda - 1| \]  \hspace{1cm} (16)

\( \Delta l \) is the deformed distance, \( l_0 \) is the original length and \( \lambda \) is the elongation, \( \lambda = l/l_0 \).

For a viscoelastic material, a stress-strain curve may look like that in figure 26. For low strains, the stress to strain ratio is constant. This is usually denoted "the linear region".
Fig. 26 Stress to strain plot for a gelatin-skim milk mixed gel. 5% w/w gelatin and 3.33% skim milk powder, salt concentration of 2.5%.

Fig. 27 (a) Tensile stress of a rectangular block and (b) shear stress of a cube. The arrow is acting normally on face $A_0$, the sample is fixed at the hatched surface (Ross-Murphy, 1984).

The force may be applied either parallel or perpendicular to the surface. See figure 27.
This is denoted tensile deformation and shear deformation, respectively. Tensile stress is usually denoted by $\sigma$ and the shear stress by $\tau$. The tensile strain is denoted by $\varepsilon$ and described above, while the shear strain is denoted by $\gamma$. In the figure a cube of the material has been deformed by shear through an angle $\alpha$, and the shear strain may be defined as

$$\gamma = \tan \alpha = \alpha$$  \hspace{1cm} (17)

when $\alpha$ is expressed in radians and when the deformation is sufficiently small.

Another important parameter is the rate of strain, defined as

$$\dot{\gamma} = \frac{\delta \gamma}{\delta t}$$  \hspace{1cm} (18)

The stress in viscoelastic materials depends upon the rate of strain.

The elasticity of a material is usually described by the elastic modulus (Young's modulus), which is denoted by $E$ in extension/compression and $G$ in shear, defined as

$$E = \frac{\sigma}{\varepsilon}$$  \hspace{1cm} (19)

$$G = \frac{\tau}{\gamma}$$  \hspace{1cm} (20)

For a viscoelastic material, the strains should be low enough for the resultant stress to strain ratio to be constant, i.e. they should be in "the linear region".

Most biopolymer gels are "energetic", which means that the response to deformations involves adaption of conformationally higher states. This adaption is the major contribution to the elasticity of a network (Ross-Murphy, 1984).

5.4.1.1 Non-destructive tests
With the help of non-destructive tests, also called dynamic viscoelastic measurements, one is able to characterize a material. The measurements may be performed during such a small degree of deformation that effects on structure are negligible. It is also possible to follow the time course of gelation, which is of great advantage. Usually, an oscillating strain or stress is applied to the sample and the response, also an oscillating strain or stress, is measured. The response from a totally elastic material is in phase with the applied strain or stress, while for a viscous liquid it is totally out of phase, i.e. 90°. For a viscoelastic material, the phase difference is between 0° and 90°. See figure 28 (Mitchell, 1980).
Fig. 28 Dynamic experiment showing responses for an elastic solid, a liquid and a viscoelastic material (Mitchell, 1980).

The ratio of in-phase stress to strain is known as the storage modulus, denoted $G'$, and the 90° out-of-phase stress to strain is the loss modulus, denoted $G''$. The storage modulus describes the elastic part of a material, and the loss modulus the viscous part. The relationship between $G'$ and $G''$ is the complex modulus, $G^*$, defined as

$$G^* = \frac{\tau_{\text{max}}}{\gamma_0} = G' + jG''$$  \hspace{1cm} (21)$$

$\delta$ is the phase difference between stress and strain, $\tau_{\text{max}}$ the maximum stress, $\gamma_0$ the
maximum strain and \( j^2 = -1 \). The phase angle \( \delta \) is related to \( G' \) and \( G'' \) as

\[
\tan \delta = \frac{G''}{G'}
\]  

By monitoring \( G' \) and \( G'' \), the development of a viscoelastic gel can be followed. See figure 29. As the network in the solution develops, both \( G' \) and \( G'' \) increase, and when network formation is finished, after 60 sec in this figure, the increase declines to a permanent level (Shoemaker, 1992).

![Graph showing G' and G'' moduli over time](image)

Fig. 29 Dynamic storage \( G' \) and loss \( G'' \) moduli of a skim-milk gel during its formation at 32°C; the milk had already undergone rennin hydrolysis at 4°C for 24 hr (Shoemaker, 1992).

An applied oscillating strain can be written as

\[
y = y_0 \sin \omega t
\]  

where \( y_0 \) is the applied maximal strain, \( \omega \) the angular frequency and \( t \) time. The stress can be written as

\[
\tau = \tau_0 \sin(\omega t + \delta) = \tau_0 \cos \delta \sin \omega t + \tau_0 \sin \delta \cos \omega t
\]
The first of the components in the equation can be interpreted as the amplitude of the component of strain in phase with the stress, and the second can be interpreted as the amplitude of the component of strain 90° out of phase with the stress. This makes it possible to express the stress in terms of the storage modulus $G'$ and the loss modulus $G''$:

$$G' = \frac{\tau_0 \cos \delta}{\gamma_0} \quad (25)$$

$$G'' = \frac{\tau_0 \sin \delta}{\gamma_0} \quad (26)$$

and

$$\tau = \gamma_0 (G' \sin \omega t + G'' \cos \omega t) = G' \tau + \frac{G''}{\omega} \dot{\gamma} \quad (27)$$

A plot where $G'$ and $G''$ are charted as functions of the frequency is known as the "mechanical spectrum". A plot like this may also define a gel rheologically, see figure 30 (Clark and Ross-Murphy, 1987). $G'$ and $G''$ of a gel with strong cross-links, for example, covalent bonds, are almost independent of frequency and $G' \gg G''$. $G''$ has a minimum in this picture. For an entanglement structure, $G'$ and $G''$ are dependent on the frequency. Another important feature of an entanglement structure is that $G'$ and $G''$ perform a crossover during a frequency sweep. At low frequencies it is clear that the entanglement structure flows, but at higher frequencies it behaves like a weak gel, or even a strong gel. For a weak gel, $G' \geq G''$. 

101
Fig. 30 Typical mechanical spectra \((G' \text{ filled circles and } G'' \text{ open circles})\) for gel (top), weak gel (centre) and entanglement network systems, plotted against frequency (Clark and Ross-Murphy, 1987).

The strain dependence of the three gels is shown in figure 31. A weak gel is only linear for small strains, while a strong gel and an entanglement gel are linear for much higher strains.

Fig. 31 Strain dependence of shear modulus for typical (a) "weak gel", (b) entanglement network and (c) gel systems (Clark and Ross-Murphy, 1987).
The elasticity is denoted $E$ in compressive and tensile tests. It is measured as the gradient, in a stress/strain curve, for very small strains. The elastic modulus of extension/compression is related to that for shear as

$$G = \frac{E}{2(1+v)}$$

(28)

where $v$ is Poisson’s ratio. $v$ being equal to 0.5 predicts that the material is incompressible or nearly incompressible, i.e. constant volume during deformation. Many biological materials are incompressible or nearly incompressible. This has generally been said to correspond to Poisson’s ratio being equal to 0.5 (Stading, 1991; Whorlow, 1980).

Dynamic tests may provide a large amount of information if they are used carefully. For example, one has to make sure that the material deforms in a linear manner for the applied stress, i.e. you have to make the measurements within the “linear region”. It is, for example, assumed that a harmonic strain results in a harmonic stress. It is also assumed that the strain is the same at all points in the material, i.e. inertial effects are negligible.

5.4.1.2 Destructive tests
Destructive tests are divided into compression and tensile tests. Compression tests are easier to perform than tensile tests due to the difficulties in fixing the sample in the testing machine. In contrast to dynamic tests, the sample is here deformed until it breaks, and the force is measured as a function of time.

In tensile tests one measures the strain or stress at fracture, whereas in compression tests one measures the hardness of the gel when compressed to a certain degree. In tensile tests it is difficult to control the location of the fracture. Fracture at the ends of the sample may give wrong results. This can be avoided by dumb-bell-shaped samples and ring-shaped samples. When measuring on a straight sample, an artificial notch can be used to direct the fracture. At the tip of the notch the stress is higher than elsewhere due to stress concentration. Materials vary in their sensitivity to notches. The stress in a viscoelastic material sensitive to notches may be related to the notch depth as

$$\sigma \propto \frac{1}{l^m}$$

(29)

where $l$ is the notch depth and $m$ a constant (Purslow, in press). The stress of a material not sensitive to notches may be expressed as in formula 30, where $\sigma_{\text{f}}$ is the stress at fracture for a sample without an applied notch and $a$ is the breadth of the
sample. The stress decreases linearly with increasing notch depth for a notch-insensitive material.

\[ \sigma = \sigma_0 (1 - \frac{t}{a}) \]  

(30)

The notch sensitivity is an important quantity providing information about the size of the largest natural structure element, \( x \), causing fracture (Birchall et al, 1981). For \( l > x \) the stress of a notch-sensitive material obeys equation 30, i.e. it behaves as if it is notch-insensitive, but for \( l < x \), equation 29 is valid. Thus, the smallest notch depth that obeys equation 29 is equal to the largest structure element \( x \), see figure 32 (Stading and Hermansson, 1991).

\[ \text{stress at fracture} \]

\[ \alpha \]

Fig.32 Stress at fracture as a function of notch size for a material that is (a) notch-sensitive and (b) not notch-sensitive. The size of the largest structure element is shown by \( x \) (Stading and Hermansson, 1991).

5.4.1.3 Viscosity

The viscosity is the most widely used property in the characterization of liquids. It is defined as

\[ \eta = \frac{\tau}{\dot{\gamma}} \]  

(31)

The viscosity can be measured by a wide range of different techniques, reviewed by Stading (1991).
To rheologists the intrinsic viscosity is an interesting quantity. It is the viscosity measured on very dilute solutions while extrapolating them to zero concentration. The intrinsic viscosity reflects the behaviour of individual polymer molecules, which is very interesting since they play an important part in gel formation, and it is usually measured in order to find out the molecular weight and conformation of a polymer (Mitchell, 1978).

The intrinsic viscosity is defined as

$$[\eta] = \frac{1}{C} \left( \frac{\eta - \eta_s}{\eta_s} \right), C \to 0$$  \hspace{1cm} (32)$$

where $\eta_s$ is the viscosity of the solvent and $\eta$ the viscosity of the solution. It is seen from the definition of intrinsic viscosity that it is expressed as a reciprocal concentration. $[\eta]$ is usually obtained by measuring the right hand side of the equation at a series of dilute concentrations and then extrapolating to zero concentration. The concentrations measured should be so dilute that no interactions between molecules are possible and, thus, able to contribute to the viscosity. Dilute solutions may show non-Newtonian behaviour, for example, for some polysaccharides, and in this case it is also necessary to extrapolate to zero rate of shear in order to measure the intrinsic viscosity.

The calculation of molecular weight from the intrinsic viscosity is dependent on the size and shape of different polymers and has been discussed (Mitchell, 1978). The relationship is usually expressed by the Mark-Houwink equation

$$[\eta] = K M^a$$  \hspace{1cm} (33)$$

where $K$ and $a$ are constants. The exponent $a$ for different molecular conformations of polymer behaviour is expressed in table 6 below.

<table>
<thead>
<tr>
<th>Model</th>
<th>$a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere</td>
<td>0</td>
</tr>
<tr>
<td>Rod</td>
<td>1.8</td>
</tr>
<tr>
<td>Random coil (equivalent sphere model)</td>
<td>0.5-0.8</td>
</tr>
<tr>
<td>Free draining random coil</td>
<td>1.0-1.2</td>
</tr>
<tr>
<td>Globular protein</td>
<td>0</td>
</tr>
<tr>
<td>Denatured globular proteins</td>
<td>0.64$^*$</td>
</tr>
</tbody>
</table>


The sphere model, having an a-value of 0, relates to compact, rigid spheres. Their intrinsic viscosity is independent of particle size and, consequently of molecular weight too (Rha and Pradipasena, 1986). The equivalent sphere model refers to a random coil polymer chain and the solvent bound to it, moving within a certain volume. The appearance of this volume may be approximated and treated like a sphere. This model is dependent on molecular weight and has an a-value between 0.5 and 0.8.

The interesting polymers in this work are globular proteins, as these constitute whey. The a-value for globular proteins, depicted in the table, falls under the sphere model category and is equal to 0. This suggests that the intrinsic viscosity of whey is independent of particle size and molecular weight. The table also shows that the a-value for denatured globular proteins is 0.64. This is similar to the value found for the random coil model (Rha and Paradipasena, 1986; Mitchell, 1978).
5.5 Viscoelastic properties of whey protein gels

As mentioned above, numerous factors affect the viscoelastic properties of whey protein gels, for example, heating conditions, protein concentration, pH, salt concentration and kind of salt, free sulfhydryl concentration, composition of whey protein concentrates etc. In this section, the effects will be discussed and one can conclude that the viscoelastic properties of gels can be altered, and that knowledge of this technique is essential if the utilization of whey is to increase.

5.5.1 Effect of heating conditions

The temperature treatment is of great importance to the properties and appearance of the formed gel, since it, to some extent, determines the gelation mechanism and the kinetics of gelation. see sections 5.1.1 and 5.1.2. Hermansson (1986) showed, for example, that a coarser structure with larger aggregates and pores was obtained at a higher temperature treatment than at a lower one.

When studying the effects of heat, one often measures the gelation temperature. Usually, gelation is marked by a change in the storage modulus, $G'$, loss modulus, $G''$, or the phase angle, $\delta$. In figure 33, the gelation of a whey protein solution is followed, with the help of $G'$, $G''$ and the phase angle, $\delta$, as a function of temperature at three different pH-values, 3.0, 5.5 and 7.5. The concentration of the solutions is varied with the pH. see figure text. The measurements were performed with the help of dynamic measurements. In figure 33a, the storage modulus is plotted against temperature. For the solution at pH 5.5, one can see that the storage modulus increases in two steps, one at a lower temperature, around 45°C, less pronounced than the increase at higher temperature, around 70°C. This result suggests that network formation starts at 45°C and develops more rapidly as the temperature increases to 70°C. These two steps can also be seen in figure 33c for the solution at pH 5.5, where the phase angle is plotted against temperature. For the gel at pH 3.0, network formation starts around 82°C and is quite rapid, whereas the network formation at pH 7.5 starts at 70°C and is slow. The difference between the gels is that, at pH 5.5, the gel is aggregated, whereas at pH 3.0 and 7.5 the gels are fine-stranded. Gelation takes place at a higher temperature for the fine-stranded gels and no two-step process is observed.
Fig. 33 Gelation of β-lactoglobulin 12% w/w at pH 3.0 (---); 10% w/w at pH 5.5 (—); 14.6% w/w at pH 7.5 (—). (a) Storage modulus $G'$; (b) loss modulus $G''$; (c) phase angle $\delta$ (Sading and Hermansson, 1990).

In figure 34 the gelation temperature, measured in three different ways, is plotted against pH for a 12% w/w β-lg solution. Comparison with the results in figure 33, despite the small difference in protein concentration, shows good agreement. Denaturation temperatures for β-lg are also depicted in the figure, as dotted lines. The figure indicates that under certain conditions, i.e. at pH-values near the isoelectric point, aggregation and gel formation may precede denaturation depending on how the gelation temperature is measured.

The gelation temperature may also be plotted as a function of heating rate, as in figure 35. The gelation temperature increases as the heating rate increases for the three pH-values studied, 2.5, 6.5 and 7.5. If the heating rate is too high, the reaction has not enough time to develop and the gelation temperature will be high.
Fig. 34 The gelation temperature, $T_g$, of 12% w/w β-lactoglobulin as a function of pH. Reported denaturation temperatures, $T_d$, of β-lactoglobulin measured by DSC are shown by dotted lines (Stading and Hermansson, 1990).

Fig. 35 The gelation temperature of 10% w/w β-lactoglobulin, measured as the point where $G'$ starts to increase, as a function of heating rate (Stading and Hermansson, 1990).

Haggett (1976) reported that the gelation temperature for whey protein is dependent on pH and salt content. It was concluded that whey protein concentrate gels at pH 8.5 have lower gelation temperatures and gel strength than those at pH 6.0. The gelling properties were not affected by whey pasteurization. See table 7. The gelation took place during heating in a water bath at a heating rate of 1.5-2.5°C. The gelation
temperature was measured as the point where a test tube of whey protein solution could be inverted without movement of the contents. The gel strength was measured by a penetrometer cone.

<table>
<thead>
<tr>
<th>Unpasteurized whey</th>
<th>Gelation temp. (°C)</th>
<th>Gel strength (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.0</td>
<td>78</td>
<td>13</td>
</tr>
<tr>
<td>pH 8.5</td>
<td>70</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pasteurized whey</th>
<th>Gelation temp. (°C)</th>
<th>Gel strength (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.0</td>
<td>79</td>
<td>12</td>
</tr>
<tr>
<td>pH 8.5</td>
<td>70</td>
<td>9</td>
</tr>
</tbody>
</table>


The small discrepancies noticed between the gelation temperatures recorded by Stading and Hermansson (1990), described above, and Haggett (1976) may be due to the difference in the experimental performance. Haggett defined the gelation point as the point when no flow was possible in a heated tube. Stading and Hermansson used small, dynamic, measurements. Another difference was that Haggett used whey protein concentrate, while Stading and Hermansson used β-lg.

The gel strength of whey protein concentrates using different heating temperatures at pH 7.0 and a concentration of 10% w/w. has been studied. It was concluded that no gel was formed at 70°C, which is in agreement with the results in figure 34 for β-lg at pH 7.0. The gelation temperature was around 85°C. The qualitative tests were performed by heating a whey protein solution for 30 min in a heating bath of constant temperature, thus allowing the solution to gel. After cooling of the sample, the gel strength was evaluated by comparison with whey protein reference gels, on a rating scale of 0-5, 0=no gel formed and 5=strong gel formed. The quantitative tests were performed by penetration of the gel in an Instron Universal Testing Instrument. The work involved in the penetration was calculated from the area under a force-distance curve and the results expressed as a percentage of the penetration data compared with some control gels. The results are depicted in table 8 (Schmidt et al., 1978b).
<table>
<thead>
<tr>
<th>Heating temperature (°C)</th>
<th>Qualitative gel strength(^a)</th>
<th>Quantitative gel strength(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>0.0</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>80</td>
<td>3.0</td>
<td>32.9a</td>
</tr>
<tr>
<td>90</td>
<td>5.0</td>
<td>85.9b</td>
</tr>
<tr>
<td>100</td>
<td>5.0</td>
<td>100.0c</td>
</tr>
<tr>
<td>110</td>
<td>5.0</td>
<td>124.0d</td>
</tr>
</tbody>
</table>

Table 8. Gel strength of 10% whey protein concentrate (WPC) heated at 70-100°C for 15 min (Schmidt et al., 1978b).

\(^a\) Mean of five replicates. (Rating scale: 0=no gel, 5=strong gel).

\(^b\) Calculated from penetration data as percentage of whey protein concentrate control heated at 100°C; Mean of four replicates.

\(^c\) Not detectable (too liquid).

The heating rate is an important factor, reflected in the gel properties. If a slow heating rate is used during gelation of a β-lg gel at pH 7.5, an inhomogeneous network may be formed with polymer-rich and polymer-poor regions, whereas a homogeneous network is formed at a faster heating rate (Stading et al., 1992). The formation of the inhomogeneities was monitored by measuring G' during gelation with the help of dynamic measurements. The G' curve proved to have a maximum at 70°C, indicating a separation into polymer-rich and polymer-poor regions in the network. The inhomogeneities were also confirmed by electron microscopy. The inhomogeneous gels had a lower G' than the homogeneous ones, and during a frequency sweep, they showed two slopes, see figure 36, caused by different relaxation times in the loose and dense regions.

Inhomogeneities in the network structure of β-lg gels have also been noticed at pH 5.3, i.e. where the network is composed of particles. As in the case of fine-stranded gels, they depended on the heating rate used. Both large and small deformations were measured on the gels. Inhomogeneities were shown in both pore size, particle size and in the occurrence of close-packed sheets. The largest spherical particles occurred at a heating rate of -1°C/min. The range in heating rate studied was from 0.1°C/min to 12°C/min. During a frequency sweep of these gels, only one slope was observed, compared to two for the fine-stranded gels. This could be due to the fact that the frequency region studied not correspond to the scale of the particulate gels studied. For the inhomogeneous particulate gels, a higher G' and a lower fracture at stress was observed. The reason for this might be the close-packed sheets, which are elastic but cause stress concentration at large deformations (Stading et al, in preparation).
Heat-induced gelation of the individual proteins in whey has been studied using dynamic measurements. BSA proved to have good gelation properties and to be highly elastic. β-lg had intermediate gelation properties and formed viscoelastic gels. α-la had poor gelation properties; in fact, it did not form a gel at all under the conditions chosen in the study referred to. It was also stated that the thermal gelation could be controlled by changes in protein concentration, pH and ionic strength. On mixing of the proteins at equal concentrations, the influence of individual proteins on thermal gelation decreased in the order BSA, β-lg, α-la. If a protein predominates in concentration, it usually determines the gelation properties. This suggests that β-lg governs the thermal gelation of whey, since it provides the largest contribution to whey. Paulsson also points out the importance of knowing the gelation behaviour of individual proteins under appropriate conditions, in order to understand the gelation pattern of a protein mixture (Paulsson et al, 1986).
5.5.2 Effect of protein concentration

A positive correlation between the square of the protein concentration and gel strength has been observed for β-lg and BSA gels at pH 8, see figure 37 for β-lg gels. The measurements were performed during compression to different degrees, 20% and 70%. A biphasic response was observed at 20% compression for both gels, around 12% protein. This behaviour may be explained by the involvement of different crosslinking mechanisms, which form a better network and harder gels above this concentration, reflected by the rise in the slope of the curve. The minimum protein concentration for gel formation of β-lg was 5% and 4.4% for BSA (Matsudomi et al, 1991a). This does not agree with the gelation concentration found in figure 21 for β-lg at pH 8, on 13%. The difference may be due to several factors, for example the measuring techniques, small deformations were performed in figure 21 and large deformations here, the heating conditions and other environmental conditions.

Fig.37 The relationship between the hardness of gels and protein concentration squared at 70 (a) and 20% (b) compression of β-lactoglobulin gels, respectively (Matsudomi et al, 1991a)
The effect of whey protein concentration on gel strength at pH 7.0 has also been studied with the help of compression (Langley and Green, 1989). They noticed an increase in compressive strength as the whey protein concentration increased and as the β-lg content in whey protein powder increased. The elastic modulus, calculated as the slope in a stress-strain plot, increased both with the whey protein concentration and with β-lg content in the same manner as the compression curves.

5.5.3 Effect of pH

It is quite clear that the pH-value affects the character of a gel to a very great extent and, of course, much research has been done in this area. The pH dependence on denaturation and aggregation has been discussed, see sections 2.6.1 and 3.2.1, respectively. The pH affects the extent of protein-protein and protein-solvent interactions by determining the charge of the protein as its ionic nature is revealed during denaturation. A carefully chosen pH-value may create a proper balance between the rate of denaturation and aggregation, and between forces of attraction and repulsion leading to the formation of a protein gel.

![Figure 38](image)

Fig.38 The storage modulus of 10% β-lactoglobulin gels after 30 min at 20°C as a function of pH (Stading and Hermansson, 1990).

The storage modulus of a 10% w/w β-lg gel, measured with the help of dynamic measurements, is plotted as a function of pH in figure 38. The β-lg solution was first heated at 90°C for 1h and subsequently cooled to 20°C. It was held there for 30
minutes before measurements took place. In the figure, two different preparations of β-lg are compared. The storage modulus is highest for the gels at intermediate pH-values where the aggregated form of the gels exists (Stading, 1990). This result is in agreement with the result from Paulsson and Dejmek (1989), who also measured the storage modulus with the help of dynamic measurements. Gels of pH 4.5, 5.0 and 7.0 were then investigated, and the gel at pH 5.0 was found to have the highest gel stiffness.

The fracture properties at different pH-values, obtained from tensile tests, have been studied in an Instron Universal Testing Machine. In figure 39, stress-strain curves for 12% w/w β-lg gels are shown, and it can be seen that there is a marked difference between them. The stress and strain at fracture are extracted from this figure and are shown separately in figure 40.

Fig. 39 Stress-strain curves in tension for 12% β-lactoglobulin gels (Stading and Hermansson, 1991).

Figure 40a may be divided into three sections, the gel formed at low pH-value is very brittle with low strain at fracture, whereas the gel formed at high pH-values can be extended to very large deformations. At intermediate pH-values, i.e. pH~ 4.5-6, the strain at fracture is constant at intermediate values. The microstructure of the gels at low and high pH-values is similar, and fine-stranded gel networks are formed. At intermediate pH-values, aggregated networks are formed. Comparing this result on large deformations with that of small deformations described above, specially figure 38, one may conclude that a difference in structure of the fine-stranded gels, formed at low and high pH, is indicated by large deformation studies, not by small. The G′
for small deformations, at low and high pH-values, is the same and no difference in structure is noted. A reason for the discrepancies when relating results from large and small deformations may be that in small deformations the whole structure of the gel contributes equally, since no parts of the structure are broken. In contrast, in large deformations, the structure is broken and the weaker areas in the network are broken before the stronger part, reflecting the structure difference (Stading and Hermansson, 1991).

Fig. 40 Strain at fracture (a) and stress at fracture (b) for 12% β-lactoglobulin gels (Stading and Hermansson, 1991).
In figure 40b, the stress at fracture is plotted against pH. The stress is low at low pH-values and increases to a maximum at pH 6.0, after which it decreases. This decrease at pH>6.0 coincides with an increased critical gel concentration at high pH, see figure 21, which is around 11.5% at pH 7.5. In the pH-range where aggregated gels form, i.e. between pH 4-6, a difference is noted. Between pH 4-5.2, the gels have lower stress than those formed between pH 5.5-6.0, indicating a difference in structure not observed with small deformations (Stading and Hermansson, 1991).

The hardness as a function of pH of β-lg and BSA gels has been studied with the help of large deformations in the form of compression to different degrees, 70% and 20%. Maximum hardness was found at a pH of 6.5 for both proteins. At pH-values below and above 6.5, the gel hardness decreased rapidly (Matsudomi et al. 1991a).

As mentioned in the section for non-destructive tests, a strong gel is almost independent of frequency, while an entanglement structure is dependent. A weak gel is dependent on the frequency. The degree of frequency dependence of a gel can be described in terms of n, defined as the slope in a log G'-log f plot, f being the frequency. The relationship between the parameters may be expressed as

\[ \log G' = n \log f + K \]

where n is the slope, G' is the storage modulus, f the oscillating frequency and K a constant (Egelandsdal et al. 1986). From the equation one can see that, in a plot of

![Graph showing the relationship between pH and n](image-url)
log $G'$ towards log $f$, the slope is $n$. For a strong gel, the slope is 0, and for a weak gel, $n > 0$. In figure 41, $n$ is plotted against pH for $\beta$-lg gels at 20°C. The $\beta$-lg concentration is varied for the gels in order to obtain equal $G'$. It is clear from the picture that this is a way of distinguishing between aggregated gels and fine-stranded gels. At low and high pH-values, where fine-stranded gels exist, $n$ is low. At intermediate pH-values, where the aggregated gels exist, the slope is higher. This suggests, when comparing with the mechanical spectra defined in section 5.4.1.1, that the fine-stranded gel is more similar to a "strong gel" with strong bonds than the aggregated one.

Fig.42 The storage modulus as a function of frequency around the upper shift between a particulate and a fine-stranded network structure for 12% $\beta$-lactoglobulin gels heated at 5°C/min. The lines are fitted to the experimental data. $\bullet$ pH 5.58, $\diamond$ pH 5.81, $^*$ pH 5.97, $\circ$ pH 6.33, $\bullet$ pH 6.50 (Stading et al, 1992).

In figure 42, the storage modulus is plotted against frequency for 12% $\beta$-lg gels. The gels were prepared at a heating rate of 5°C/min at different pH-values around the upper shift in microstructure. The gel at pH 5.58 had the highest $G'$ and was almost
independent of frequency. The gel formed an aggregated network at this pH-value. At pH 6.5, the slope is steeper. The gels formed at this pH-value have fine-stranded networks. The frequency dependence of the gels at pH-values in between these two described showed two slopes, indicating inhomogeneities in the network structure. The two-slope behaviour may, in this case, depend upon the network structure, which shifts from an aggregated network to a fine-stranded network in this pH-interval (Stading et al, 1992). It was shown before that the inhomogeneities may arise from too slow a heating rate. This is not the reason in this case, as a constant heating rate was used. See section 5.5.1 regarding the inhomogeneities in gels. The inhomogeneities at this shift have been verified further with the help of transmission electron microscopy (Langton and Hermansson, 1992).

The slope is higher for the fine-stranded gels than for the aggregated gels. However, in figure 42, which is in direct contradiction to the result obtained in figure 41, this behaviour may be easily explained by the fact that the β-lg concentration in figure 42 is the same for all gels. Looking in figure 21, the critical β-lg concentration for gel formation is plotted against pH. It is clear from the picture that the critical gel concentration for the fine-stranded gel at pH 6.5 is close to 12%, indicating that the concentration studied in figure 42 is near the critical concentration for gel formation. In contrast, for the aggregated gel at pH 5.58, the critical gel concentration is much lower, i.e. around 1%, indicating that a stronger network is formed, since gel strength is concentration-dependent.

5.5.4 Effect of salt

As mentioned before, the ability of a whey protein solution to form a gel depends upon the critical balance between attractive and repulsive forces. This balance may be altered by the addition of salt, which starts a complex reaction. At a low salt concentration, the primary effect of ions on protein structure is their electrostatic interaction with the counter charges on the protein (Damodaran and Kinsella, 1982). By changing the salt content and perhaps altering the type of salt, changes in the gelling properties of proteins may be induced (Schmidt et al, 1979). On addition of an excessively high salt concentration, repulsive interactions may result in the collapse of the network into large aggregates surrounded by the aqueous medium.

The effect of salt on the rheological properties of whey protein gels at pH 7.0 has been examined. It was concluded that addition of low levels of NaCl gave gels with low shear stress and high shear strain values, measured with the help of large
deformations, by twisting the gel sample until break. Addition of low levels of CaCl$_2$ gave gels with low shear stress and low shear strain values. The obtained shear stress values of the whey protein gels were very similar, while the shear strain values differed greatly, indicating that shear strain is greatly dependent on the charge of the cation of the added salt.

In figure 43, the shear stress at failure for both monovalent and divalent cations is shown, and in figure 44, shear strain at failure is shown for the same ions. It is clear that the effect of divalent ions is larger than that for monovalent ions. The conclusions were that salt may be used to alter the strength (shear stress) and deformability (shear strain) of whey protein gels (Kuhn and Foegeding, 1991b).

![SALT](image)

Fig. 43 Effect of salts of monovalent and divalent cations at equal ionic strength (90 mM) on the shear stress at failure of 10% whey protein isolate gels (Kuhn and Foegeding, 1991b).

There exists an optimum amount of salt addition in order to obtain the maximum gel strength. For calcium, maximum compressive strength is obtained, for a 10% w/w dialyzed whey protein gel at pH 7.0, at a concentration of 11.1 mM. Further addition decreases the gel strength (Schmidt et al, 1979). Addition of CaCl$_2$ or NaCl to dialyzed whey protein concentrate increases its gel strength more than salt addition to a non-dialyzed whey protein concentrate (Schmidt et al, 1978a).
Fig. 44 Effect of salts of monovalent and divalent cations at equal ionic strength (90 mm) on the shear strain at failure of 10% whey protein isolate gels (Kuhn and Foegeding, 1991b).

In figure 45, the hardness as a function of calcium concentration is plotted. The optimum exists around 10 mM CaCl₂-concentration. On further increase in Ca-concentration, a clear decrease in gel hardness is obtained.

A similar investigation was performed by Mulvihill and Kinsella (1988). A maximum compressive strength was then obtained at a calcium concentration of 10 mM in a 8.33% w/w β-lg gel at pH 8.0. In this investigation, the NaCl concentration was found to produce maximum compressive strength at a concentration of 200 mM.

Quite different results were obtained by Matsudomi et al (1991a). The hardness of 10% β-lg and BSA gels was studied by compression to 70%, at pH 8. On addition of NaCl, the hardness of BSA gels was not affected, whereas the hardness for β-lg gels showed a maximum at a NaCl concentration of 20mM instead of a concentration of 200mM obtained above. The hardness of both BSA and β-lg was affected by addition of CaCl₂. The maximum hardness for BSA gels was obtained at CaCl₂ concentration of 5 mM and for β-lg, at 2 mM.
Fig. 45 Effect of CaCl₂ or cysteine addition to dialyzed 10% whey protein dispersions at pH 7.0 on hardness of gels prepared by heating at 100°C for 15 minutes (Schmidt et al., 1979).

The values obtained by Matsudomi were low in comparison with those obtained by Mulvihill and Kinsella. The heating conditions used for gel preparation were found to differ. The compression degree was also a little bit higher for the gels studied by Matsudomi. And, of course, the batches used for the experiments were not the same, which would account for some differences in the results.
Zirbel and Kinsella (1988a) found a maximum in hardness for 20% whey protein isolate gels at pH 7.0 at a CaCl₂ concentration of 20 mM. The hardness was also measured by maximum compressive strength in this case.

The association of calcium with α-la and β-lg has been studied. The amount of protein-associated Ca was determined with the help of the difference in Ca content in an ultrafiltrated retentate and an ultrafiltrated permeate. The extent of the associations were pH-dependent and increased as the pH increased, following the increase in negative charge of the protein. For α-la, protein-calcium association began at pH 4. Below pH 4 no protein-calcium association was detectable. For β-lg the protein-calcium association began at pH 5. The α-la capability to interact with calcium was almost twice as high as that of β-lg. The increase in ionic strength had a negative effect on the protein-calcium association. In conclusion, whey protein-calcium association seems to follow three distinctive patterns: no association interactions below pH 4; limited interactions between pH 4 and 5.5 due only to α-la; significant associations above pH 5.5 due to both α-la and β-lg (Patocka and Jelen, 1991). Calcium association with α-la and β-lg has also been discussed in section 5.2.4.

The effect of different anions on the rheological properties of whey protein gels has been studied by compression measurements. Two protein-structuring anions, Cl⁻ and SO₄²⁻, were compared with one destructuring anion, SCN⁻. The ions proved to enhance gel strength in the order SCN⁻>Cl⁻>SO₄²⁻, above concentrations of 100 mM. The hardness of the gels increased with increasing concentrations up to 0.1, 0.2 and 1.0 mol/dm³ with SO₄²⁻, Cl⁻ and SCN⁻ respectively (Mulvihiill et al, 1990).

<table>
<thead>
<tr>
<th>Anion</th>
<th>Conc (mM)</th>
<th>Gel hardness (N) BSA</th>
<th>Gel hardness (N) β-lg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO₄²⁻</td>
<td>50</td>
<td>4.86</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5.13</td>
<td>0.51</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>50</td>
<td>4.51</td>
<td>3.83</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.52</td>
<td>2.10</td>
</tr>
<tr>
<td>Br⁻</td>
<td>50</td>
<td>4.34</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.30</td>
<td>2.31</td>
</tr>
<tr>
<td>I⁻</td>
<td>50</td>
<td>4.23</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.23</td>
<td>2.53</td>
</tr>
<tr>
<td>SCN⁻</td>
<td>50</td>
<td>4.20</td>
<td>3.80</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.14</td>
<td>3.65</td>
</tr>
</tbody>
</table>

Table 9. Effect of anions on gel hardness of BSA and β-lg at 70% compression (Matsudomi et al, 1991a).
The effect of different anions on gel hardness has also been studied by Matsudomi et al. (1991a) with the help of 70% compression measurements, see table 9. All samples in the table have been prepared with the sodium salt at pH 8.0 and a protein concentration of 10% w/v.

When comparing table 9 with the results for whey protein gels obtained above by Mulvihill et al. (1990), one has to restrict the resemblance to the hardness for β-lg gels, since the properties of whey are governed by the properties of β-lg. Thus, the results obtained resemble one another, the order of enhancement in gel strength being SCN\textsuperscript{-}>Cl\textsuperscript{-}>SO\textsubscript{4}\textsuperscript{2-}.

5.5.5 Effect of different environmental factors

Sulphydryl reagents, such as cysteine, can be used to increase protein gel strength through mercaptan interchange reactions. A reaction mechanism for network formation through mercaptan interchange reactions was proposed by Huggins et al. (1951). It was also pointed out that a critical mercaptan concentration exists, above which the gel network is broken by disulphide cleavage.

A maximum in gel hardness, measured by compression in an Instron Universal Testing machine, was found at a cysteine concentration of 9.7 mM in a 10% dialyzed whey protein solution at pH 7.0, see figure 45 (Schmidt et al., 1979).

In another study, the hardness of non-dialyzed whey protein gels of 10% at pH 7.0 was calculated using penetration in an Instron Universal Testing Machine. The maximum hardness was found at a cysteine addition of 25 mM. On further increase in concentration, a rapid decrease in gel hardness was found (Schmidt et al., 1978b). This behaviour can be seen from table 10.

These results lead to the conclusion that cysteine is more effective in the less complex dialyzed environment, i.e. a maximum hardness of non-dialyzed whey protein gels was found at a cysteine concentration of 25 mM compared to 9.7 mM for a dialyzed environment. It seems as if there exists an optimum concentration of sulphydryl reagents to obtain the maximum gel strength, and that this concentration depends on the nature of the protein and on the environment.

Contrary to the results presented in table 10, Kohnhorst and Mangino (1985), found that sulphydryl reagents were not good predictors of whey protein gel strength. Only a small effect was noticed. The gels had a whey protein concentration of 7.5% and
a pH of 4.6. The gels used were obtained from different membrane processed whey protein concentrates, and penetration measurements were used.

| Cysteine (mM) | Qualitative gel strength | Quantitative gel strength
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.0</td>
<td>100.0a</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>203.0b</td>
</tr>
<tr>
<td>10</td>
<td>5.0</td>
<td>211.5b</td>
</tr>
<tr>
<td>25</td>
<td>5.0</td>
<td>328.3c</td>
</tr>
<tr>
<td>75</td>
<td>5.0</td>
<td>117.3a</td>
</tr>
<tr>
<td>100</td>
<td>2.0</td>
<td>15.4d</td>
</tr>
</tbody>
</table>

Table 10. Cysteine effect on gel strength of 10% whey protein concentrate heated at 100°C for 15 min (Schmidt et al, 1978b).

* Mean of five replicates. (Rating scale: 0=no gel, 5=strong gel).

* Calculated from penetration data as percentage of whey protein concentrate controls heated at 100°C: Mean of four replicates. Means followed by the same letters are not significantly different (P<0.05).

The difference reported concerning the importance of sulfhydryl reagents to gel strength may be due to the different pH-values used on the gels. This is in agreement with the discussion in the section 5.2.2 about covalent forces, which pointed out that the SH-activity is pH-dependent. At low pH-values, i.e. pH 4.6, the SH groups are relatively inert and do not contribute to gel strength, whereas at high pH-values, i.e. at pH 7.0, the SH groups are active, and a good correlation exists between SH-content and gel strength.

<table>
<thead>
<tr>
<th>Conc. of reagent (mM)</th>
<th>NEM added to BSA</th>
<th>NEM added to β-lg</th>
<th>DTT added to BSA</th>
<th>DTT added to β-lg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>4.655</td>
<td>3.346</td>
<td>4.655</td>
<td>3.350</td>
</tr>
<tr>
<td>2.0</td>
<td>3.026</td>
<td>3.500</td>
<td>6.001</td>
<td>4.500</td>
</tr>
<tr>
<td>5.0</td>
<td>0.766</td>
<td>3.601</td>
<td>6.381</td>
<td>0.724</td>
</tr>
<tr>
<td>10.0</td>
<td>0.247</td>
<td>3.051</td>
<td>5.252</td>
<td>0.701</td>
</tr>
<tr>
<td>20.0</td>
<td>0.202</td>
<td>0.170</td>
<td>2.287</td>
<td>0.683</td>
</tr>
<tr>
<td>50.0</td>
<td>0.149</td>
<td>0.090</td>
<td>2.640</td>
<td>0.371</td>
</tr>
</tbody>
</table>

Table 11. Effect of thiol reagents N-ethylmaleimide and dithiothreitol on the hardness (N) of gels formed from β-lactoglobulin and Bovine Serum Albumin (Matsudomi et al, 1991a).
Matsudomi et al (1991a) studied the effect of thiol reagents on the hardness of BSA and β-lg gels made with 10% w/v protein at pH 8.0. β-lg has one thiol group and two disulphide groups per monomer and BSA has one thiol group and 17 disulphide bonds. The hardness was measured with 70% compression of the gels formed at 90°C for 15 min as the concentration of N-ethylmaleimide (NEM) or dithiothreitol (DTT) was changed. See table 11.

On addition of NEM, the free thiol group is blocked and it is hindered from taking part in the thiol-disulphide interchange reactions between protein molecules. The hardness of BSA decreased markedly on addition of NEM over 2 mM, while for β-lg, an addition of 20 mM was required to markedly decrease the hardness, suggesting that the BSA gel was more sensitive to blockage of the thiol group than β-lg. The reason for this behaviour is not obvious, since the single free thiol group should be equally accessible to the reagent (Matsudomi et al, 1991a).

On addition of DTT, disulphide bonds are reduced. The hardness of BSA on addition of DTT increased to a concentration of 5 mM, see table 11, and then decreased on further addition. The addition of DTT to β-lg had a similar effect. The gel hardness first increased to a DTT concentration of 5 mM, and on further addition it decreased. The initial increase in hardness of the gels on addition of DTT may be due to the increased facility in unfolding of the protein as the disulphide bonds are reduced, favouring network formation. Extensive reduction of disulphide bonds may take place on further addition, resulting in excessive interactions due to the excessive unfolding, coagula being formed instead of structured gel network (Matsudomi et al, 1991a).

The effect of thiol reagents and ethanol on the gel strength of whey protein has been studied with the help of compression tests. The gels contained a protein concentration of 20% and a pH-value of 7.0. On addition of p-hydroxymercuribenzoate (pHMB) and NEM over 4 mmol/dm³, the hardness of the gels decreased. The explanation for this was inhibition of the formation of disulphide bonds between proteins. For DTT, a concentration of 8 mmol/dm³ was needed in order to decrease the hardness. In this study, the effect of addition of ethanol was studied. As ethanol is added, the dielectric constant of the medium decreases, which strengthens electrostatic attractions between proteins (Creighton, 1983). The hardness increased as the concentration of ethanol increased, suggesting that electrostatic and hydrogen bonds are involved in the gel network (Zirbel and Kinsella, 1988b).

Mulvihill et al (1991) studied the effects of different environmental factors on the rheological properties of β-lg gels. The concentration of the gels studied was 10%, and they had a pH-value of 8 and, some gels, contained a NaCl concentration of 200
mM whereas other gels were salt free. In the absence of NaCl, no self-supporting gels were formed, due to the high energy barrier, creating excessive repulsive forces strong enough to inhibit gel formation. On addition of N-ethylmaleimide and mercaptoethanol to these NaCl free solutions, still no network was formed upon heating. The probable reason for this is that, under these conditions, they did not influence the electrostatic repulsions existing due to the high pH-value. However, on addition of propylene glycol, weak gels formed, and these increased in gel strength as the concentration of propylene glycol increased. The effect of propylene glycol was a lowering of the dielectric constant of the solvent, which reduce the energy barrier to protein-protein interactions enough to enable network formation.

An investigation was performed on the effects of shear treatments on suspensions of whey protein. It was concluded that sheared whey protein suspensions gelled more rapidly and formed stronger gels. These properties developed by shear treatment were examined when adding sheared whey protein isolate to low-fat frankfurters. The product then became firmer and the moisture loss during cooking was lower. Microscopy studies, scanning electron microscopy, were also performed on the gels. They showed a development of a fine fibrous-like structure in the sheared suspensions, indicating that mechanical shearing prior to heating accelerated protein-protein aggregation. It was further concluded that optimum time and temperature exist regarding the shear treatment, where the gel shows maximum strength. These optimum points remain to be determined (Ker and Toledo, 1992).

Commercially available whey products can show different functional properties. This was discussed to some extent in section 5.3.2. A spray drier is often used in order to obtain the whey products, and this technique may cause particle temperatures above 100°C resulting in partial denaturation and progressive polymerization of the proteins. Storage of whey products may also cause a decrease in solubility. Rector et al (1991) studied the changes in the gelling behaviour of whey protein isolate and β-lg during dry heat treatment and storage. The proteins were stored at 80°C for 7 days. This resulted in a decrease in the hardness of the gels showed by compression tests. The gels were formed from a 12% solution at pH 8.0. It was shown by gel electrophoresis that the monomeric β-lg concentration decreased from 60.64% to 33.33% after 7 days storage at 80°C. The polymerization did not involve disulphide bonds.

The effect of different molecular weights on the hardness of various globular proteins has been studied. It is clear from the results that a linear relationship exists between gel hardness, measured as compression, and molecular size (and shape). It is also pointed out that disulphide bonds do not contribute to the gelation of proteins but rather to the ability to increase the molecular weight of the protein (Wang and
Damodoran, 1990). A lot of research has been done on the importance of disulphide bonds to gel formation and gel strength and contradictions exist among the existing theories.
6. APPLICATIONS IN THE PATENT LITERATURE

In recent years, there has been a lot of research on low-fat products in order to improve their character, decrease their fat content even more and to develop new products. Fat-rich foods are popular and make up a significant proportion of the diets for many people. The undesirable nutritional impact and subsequent problems related to the consumption of these popular types of food products are widely recognized. This is the main reason for the increasing interest in low-fat products.

It is known that low-fat products can be produced by substituting the fat phase with an aqueous phase, consisting of milk proteins. In early stages of this field, oil-in-water emulsions, having a low-fat content and a spreadable character, were produced by incubating milk with milk acid thermobacteria. See, for example, patent nr. 359222. A main drawback of this procedure is that the material requires extra processing steps in the form of incubation. This process is intimately linked to cheese-making, and the resulting emulsion is, of course, limited to products having a cheese-like character. Later, technical improvements made it possible for spreadable products having the desired characteristics to be obtained in a considerably easier way, starting from non-modified non-fat milk solids.

A lot of different approaches have been made to develop low-fat products using whey proteins as fat replacers. The latest concerns particles, in the range of 0.1-2 microns. see section 2.2. It has been claimed that spherical particles of denatured whey proteins, in this range, produce a desirable organoleptic character and that they may substitute fat. Above 2 microns, the dispersions impart an undesirable chalky mouth feel to foods, and if less than 0.1 microns they make food taste greasy. The food industry needs improved processes for producing suitably sized and shaped protein microparticles for fat replacement. Optimally, such an improved process would diminish or eliminate the need for any substantial amounts of processing aids, such as aggregate blocking agents, in the soluble and coagulable protein solution feedstock. However, this is not an easy task since the aggregation mechanism of whey protein is unclear.

Patent applicants have found that when whey proteins are used as fat substitutes in spreads the sensation of creaminess is enhanced. The patents studied here refer specially to whey proteins. They are divided into two sections. one describes whey proteins in the form of emulsions and the other part describes the particles discussed above.
6.1 Emulsions

The patents described here refer to emulsions of whey protein. The emulsions are used as fat substitutes in low-fat products and margarines. The processes vary pH, protein and salt concentration, heating and homogenization conditions, etc. in order to obtain the desired fat consistency of the emulsion.

**Swedish patent 359 222, Cox et al, 1973**

"Invention for producing a spreadable milk product"

Cream-like, spreadable milk products are produced by a dispersion containing milk protein, for example, whey-protein, and lactose. The dispersion is incubated with thermobacteria at 43-45°C, and then cooled to about 20°C when the pH-value has reached 4.8-5.2. The fat is homogenized into the aqueous dispersion, either before or after the incubation, and an emulsion is obtained. The resulting emulsion is pasteurized at 80°C for 20 seconds, cooled and mechanically worked in a temperature intervall between 0°C and 55°C. A cream-like, spreadable product is formed. The invention is characterized by not separating the whey from the curd and by the fact that after pasteurisation, during cooling, the emulsion is mechanically worked between temperatures of 0°C and 55°C. The emulsion contains preferably between 20-75% (w/w) total dry material, 0.5-25 % (w/w) protein and 15-70% (w/w) fat.

**Swedish patent 393 2, Strinning, 1977**

"Invention for producing a low-calory margarine, comprising an aqueous phase resulting from unsoured milk"

The invention relates to the production of a low-fat margarine with a high content of proteins, for example, whey proteins. The aqueous phase consists of milk proteins, such as skim-milk, whole-milk, sweet whey or blends of them with a pH higher than 5.8. The aqueous phase is membrane-filtrated and a protein concentrate, with a protein content of 9-24%, is produced. This protein concentrate is heated between 90 and 95°C for 1 minute and 10 seconds to denaturate the proteins in a way that will increase their water-binding and emulsifying ability. The aqueous phase is then added to the fat phase and an emulsion is formed. The spread comprises between 35-65% fat, and the remainder is the aqueous phase consisting of between 9-24% protein.
This patent describes a process for producing an o/w/o double emulsion, which on consumption gives a creamy sensation and a good flavour impact on the palate. The invention relates to an emulsion containing less than 80% fat, particularly a low-calorie spread with a level of fat ranging ideally from 30-50%, and which is stable and retains its double emulsion character during storage. In this patent whey protein is used for different stabilizing purposes in an emulsion, consisting of a cream and a fat phase. The use of whey proteins allows the spread to have a pH of about 3.5 to 5.5, which promotes bacteriological stability. It also raises the viscosity of the cream, after denaturation and heat treatment. It stabilizes the fat globules in the cream and also the final cream in the fat spread. In the emulsion the continuous phase is an oil or fat, and the dispersed phase consists of a natural or artificial cream. The diameter of the cream drops generally ranges from 10 to 40 microns in the emulsion. The cream comprises heat-denatured whey protein, an oil which is present as droplets ranging from 0.1 to 2 microns, and an appropriate amount of a thickening agent. In this invention, the whey protein is preferably obtained by membrane filtration of whey, retaining the protein present in the native state in the initial stage of the process.

The process for producing low-calorie spreads is as follows:

i) producing a cream comprising a protein essentially consisting of whey protein, an oil which is present as droplets of an average diameter ranging from 0.1 to 2 microns, and an appropriate amount of a thickening agent

ii) heating the cream to denature the protein at around 85-90°C.

iii) emulsifying the cream in a fat phase

iv) subjecting the o/w/o emulsion to cooling and texturizing treatments to obtain a plastic spread.

In this patent the low-fat spread consists ideally of 30 to 50 w% fat, 0.1 to 2 wt% whey protein and 0.05 to 5 wt% thickening agent. The pH of the aqueous phase is in the ranges 3.5-5.5.

The patent also includes examples. I have chosen one to exemplify the method:

Whey protein (1.66%) obtained by membrane filtration was dissolved in water (37 wt%) at 60°C. A fat blend (7.5 wt%) at 60°C, containing flavour, was added to the protein solution and the mixture was subjected to high pressure homogenisation at 250 kg/cm² to produce a cream in which the average size of the fat droplets was about 1 micron. Gelatin (1.5 wt%), dissolved in water (15 wt%), was added to the cream. More water was added to reach a level of 55.5 wt%. The pH was adjusted
to 4.7, using a 20% solution of lactic acid and water-soluble flavours (0.2 wt %) were added. The cream was pasteurized at 90°C for 30 minutes to denaturate the whey protein. The cream was also rehomogenized at a pressure of 70 kg/cm² in order to disperse possible clumps. 32.18 wt % of the same fat blend used for preparing the cream was melted, and to the molten blend was added 0.5 wt % of fat-soluble flavours and a mixture of 0.2 % monoglyceride and 0.03 % lecithin. The blend was subsequently cooled to 40°C in a holding tank and was fed into a crystallizer, a so-called C-unit. The pasteurized cream was allowed to cool to 40°C. The cream was then proportioned in the C-unit, and the emulsion was allowed to cool to 20-25°C while being rotated at 200 rpm. The fat-continuous emulsion emerging from the C-unit was processed through a Votator sequence ACAC to obtain the final fat-continuous spread.

**US patent 4,820,541, Haring, 1989**

"Spread containing dairy fat"

The invention relates to a spread containing a dairy emulsion and optionally non-dairy fat with an improved butter-like taste and flavour. The invention provides a spread having a dairy fat content between 9 and 65% (w/w). The spread consists of a heat-treated dairy cream containing at least 30% (w/w) of dairy fat and a substantial amount of denatured whey protein. The amount of denatured whey protein is preferably 70% (w/w) calculated on the total whey protein content. The dairy cream should be heated at a temperature around 70°C for at least 20 minutes. The spread comprises between 25-90% (w/w) water and non-dairy fat. The ingredients are worked into a spread in, for example, a scraped drum cooling device (Votator R) such as used in the margarine industry. Spreads obtained from a heat treated dairy emulsion, containing whey protein, has an improved buttery taste and flavour.

**European Patent Application 0,340,857, Bos, 1989**

"A process for preparing an edible and spreadable oil-in-water emulsion"

The invention relates to an edible and spreadable oil-in-water emulsion on the basis of dairy materials. This emulsion is formed by mixing a fat-phase with a non-fat milk aqueous phase, containing a whey protein concentrate and a melting salt. The fat content of the product is preferably between 15-45 wt % and the solid content of the aqueous phase between 30-50 wt %. The milk protein content of the aqueous phase is between 8-20 wt %, of which 10-40 wt % consists of whey protein concentrate. The emulsion also contains 1-4 wt % melting salt. The melting salt is added to give
the whey protein get the correct character. The pH of the examples depicted are around 5.6. The composition of the solids of the aqueous phase is decisive of the structure of the final product. The whey protein is added in an undenatured form and is denatured in the process to bind water in the form of a gel having the correct spreadable structure of a fat. The preparation of the products is very simple and only consists in mixing the aqueous and fat phases, pasteurizing or sterilizing the resultant emulsion, homogenizing and cooling.

US patent 4 978 553, Silver, 1990 and
European Patent Application 0 390 947, Silver, 1990
"Method of making low-fat butter or margarine spread and resulting product"

This invention relates to a method for the production of a smooth, homogeneous, spreadable, protein-enriched, low-fat butter or margarine for use as a table spread. It is based on the discovery that a low-fat spreadable butter or margarine can be prepared from a dairy base, without the need for emulsifiers and stabilizers, by subjecting the ingredients to a high speed cutting action in order to rupture the fat globules. The protein is a denatured dairy protein, for example, whey protein, and is added in an amount sufficient to emulsify the fat in the product, about 8%, and less than the amount of protein which stabilizes the fat globule membrane against rupturing under plasticizing conditions, about 14%. The dairy proteins are believed to bind to the fat at the interface of the fat and protein. The action causes formation of a stable oil in protein and water emulsion. The dairy proteins also have a water binding ability which enables them to bind the free water that is in the low fat emulsion. The high speed cutting of the proteins and fats increases the surface area of the fat particles, giving more surface area for the protein to react with and bind to. This protein-fat interaction forms a stable emulsion without the addition of stabilizers and emulsifiers. The cutting action is carried out in commercially available processing equipment for high speed rotation, fitted with sharp cutting blades. The dairy protein content of the product is between 8% and 14%, the fat content between 15% and 50% and the moisture content between 40% and 60%. The admixture may also contain salt, butter colouring and butter flavouring. The addition of 5% modified potato starch allows the low-fat butter product to be freeze-thaw stable.
The invention relates to low-fat spreads containing native, that is, not substantially denatured, whey protein. Undenatured whey protein lead to improved spreads by way of its thickening effect and particular organoleptic properties. The product is a water-in-oil emulsion spread comprising from 35-80 % wt of a fat phase and from 20-65 % wt of a dispersed water phase with a pH from 4.5-5.5 and consisting of 0.5-10.0 % wt of a substantially dissolved and undenatured whey protein, wherein the lactose content of the water phase is 0-20 % wt of the total protein content. The process for the production of a spread comprises admixing an aqueous phase, containing undenatured whey protein, with a fatty phase and cooling and working the mixture until an edible fat-continuous emulsion results. The aqueous ingredients of the spread are pasteurised by heat treatment at a temperature of 65-80°C for a period of between thirty seconds and ten minutes. This slight pasteurisation is sufficient to prevent microbiological spoilage without substantially denaturing the protein. The advantage of undenatured whey protein is the avoidance of "mealinness" and the expression of additional functional properties, such as water-binding ability and some effects as an emulsifier. Another advantage is that keeping the product in this pH-range, improves long-term resistance to microbiological spoilage.

Swedish patent 461 762, Larsson and Johansson, 1990
"Invention for producing low-fat margarine, spreadable at low temperatures"

The invention relates to a process for producing a low-fat margarine with a fat content between 19-38% and a good taste and consistence. It is spreadable at refrigerator temperatures and at room temperature. The product can be pasteurized without breaking the stable emulsion. The product is also microbiologically stable for a long period. The product comprises a fat phase and an aqueous phase. The fat phase consists of 19-38% fat and emulsifiers. The aqueous phase consists of 55-60% milk protein concentrate, whey proteins included, with a protein content of 12-14%. It also contains monoglycerides, skim-milk, starch and taste and color additives. The starch contributes, to a great extent, to the desired and stable emulsion.

The process for producing the product includes five main steps:

- Production of a solution containing starch and monoglycerides. The starch is dissolved during agitation in, for example, a liquid whey solution. It is heated and allowed to stand for 24 hours.
- Production of an aqueous phase consisting of 12-14% protein, whey protein
included, from a milk product. The pH is between 6.2-6.6 and the temperature is between 47-50°C. The starch solution is added to this aqueous phase during agitation.

- Production of a fat phase with a temperature between 48-58°C consisting of flavour and colour additives.
- Production of an water-in-oil emulsion. The aqueous phase is subsequently mixed with the fat phase during harsh mixing conditions.
- Pasteurization of the emulsion around 80°C and then cooling it to between 13-8°C. The emulsion is then texturized in a texturizer.

The resulting product has the above-mentioned characteristics.


"A process for preparing low-calorie butter products and products obtained"

The invention relates to a process for preparing low-calorie butter or low-fat margarine on the basis of a fat phase from butterfat or mixtures of butterfat and other fats and an acidified aqueous phase containing milk proteins and thickeners. A low-calorie butter with an improved taste like butter is obtained when an aqueous phase is emulsified in butter obtained by phase reversal of cream. The aqueous phase should be obtained by mixing a pasteurized solution of milk protein and protein- or carbohydrate-containing thickeners with a mixture of lactic acid concentrate. The lactic acid concentrate should be obtained by bacteriological acidification of a milk product, whey proteins included, with a flavour starter. The concentrate has a pH below 4.0. The use of whey protein in the flavour culture proves to be significant to the taste impression.
The patents described here refer to specially sized particles. They are obtained by denaturation and aggregation of whey protein, and the special sizes are obtained by varying the heating and shearing conditions, pH, protein and salt concentration, etc.

**US patent 4 734 287, Singer et al. 1988**

"Protein product base"

This patent concerns a proteinaceous, water-dispersible macrocolloid comprising substantially non-aggregated particles of heat-denatured dairy whey protein. These colloids have a smooth, emulsion-like organoleptic character when hydrated. A process for the preparation of the macrocolloid is also disclosed. The product is usually called "Simplesse". The diameter of the particles ranges from about 0.1 microns to about 2.0 microns, or equivalent to a volume of about $5 \times 10^{-4}$ cubic microns to about 5.5 cubic microns. Within this particle range the colloids have an emulsion-like mouth feel. The majority of the particles are spherical. This form and size of the particles are obtained during a controlled or extent-limited heat denaturation process during which undenatured whey proteins are subjected to a high shear force to prevent the formation of any significant amounts of large whey protein aggregates. The temperature is between 80°C and 130°C and pH is between 3.5 and 5.0. The process is carried out for a time sufficient to produce a substantial amount of denatured, proteinaceous macrocolloidal particles in the range described above.

Fig.46 Processor for production of μ-particles of protein (Singer et al. 1988).
The process is carried out in a machine shown in figure 46. For a detailed description of this, see US patent 4 734 287. The above-described macrocolloid is advantageous in that it is denatured and therefore readily digestable, while retaining a high Protein Efficiency Ratio (PER), since the sulphur-containing amino acids are not lost in processing. The colloid forms gravitationally stable dispersions which are not gritty. It is therefore highly desirable as a protein supplement in human foods and has an emulsion-like organoleptic character approximating that associated with oily and fatty foods. It is therefore useful as a high-protein, low-calorie "fat replacer" in such applications. Two main products have been developed, one consisting of pure whey protein and the other of a mixture of albumen, skim-milk, sugar, pectin and citric acid.

The patent also includes a lot of examples, for instance mayonnaise and nutella, a sweet, hazel nut-chocolate sandwich spread. One example is given below to show the method for producing the macrocolloids:

A mixture was prepared comprising 41% by weight a whey protein concentrate and 44% water at 65°C. The mixture was acidified to a pH of 4.2. 30,000 units of a commercial fungal-lactase was added to the mixture. 3% by weight of lecithin was added, and the mixture was dewatered in a Versator TM running at 3.7 kilograms per minute, then permitted to stand overnight. The mixture had a specific gravity of 1.16. After standing, the mixture was then passed to a fluid-processing device, shown in figure 46, which operated under steady-state conditions wherein the rotor was run at about 900 rpm and the temperature of the heat transfer medium, steam in this case, was about 120°C at the inlet and about 117°C at the outlet. The mixture was maintained at about 80 to 90 psi during heating to prevent out-gassing of liquids, which would otherwise boil at such temperatures under ambient atmospheric pressures. The product was cooled in a single-blade, scraped heat exchange apparatus, operating at about 200 rpm, to about 80°C or less. The product was judged to be organoleptically satisfactory.

"Proteinaceous fat substitute"

The present invention provides a novel process to produce denatured whey protein microparticles, characterized by heating an aqueous whey protein solution under high shear conditions, at a pH value greater than the midpoint of the isoelectric curve of the proteins in the aqueous solution, that is, between pH 5.8-6.9. The shear conditions are maintained for a time sufficient to avoid the formation of any substantial amount of fused particulate proteinaceous aggregates having diameters in excess of about 2
microns, while also forming denatured proteinaceous macrocolloidal particles which are greater than about 0.1 microns in diameter. Within this pH-range, 5.8-6.9, the undesired aggregation of microparticles is avoided and the need for additional processing aids, such as aggregate blocking agents, is significantly reduced or eliminated. The off-flavours and odours usually obtained are also reduced or eliminated. These usually arise when aqueous whey protein solutions are used as the feedstock for microparticle formation. This pH ensures the formation of properly sized particle populations, thereby providing an improved product. The aqueous phase comprises 18-25 % by weight soluble, undenatured whey protein, and the solution is subjected to elevated temperatures for varying periods of time under shear rates which may be as high as or greater than 40,000 reciprocal seconds. Typical processing temperatures range from about 68-120°C, and processing times range from about 3 seconds to about 30 minutes or longer. The process equipment used in this case is identical to that provided by Singer, et al., US patent 4 734 287, shown in figure 46.

European Patent Application 0 347 237, Visser, 1989
"Edible plastic compositions"

This patent describes a process for the preparation of a plastic food composition. The process involves subjecting an aqueous phase consisting of heat denaturable whey protein to a succession of heating and concentration steps. The protein should preferably be heated at a concentration below the critical gel concentration, i.e. approximately below 8%. Due to these heating and concentrating steps the whey protein partly denatures and, at least, doubles its concentration. The remaining undenatured protein is removed and a plastic composition is obtained. It comprises at least 5 wt% of the heat-denatured protein in the form of non-aggregated protein-based microcolloidal particles in the range of 0.1 to 50 microns. These particles exhibit a desirable organoleptic character when dispersed in an aqueous medium and hydrated, closely related to that of fat/water plastic emulsions. The heating takes place in the temperature range of 60 to 75°C for between 1 to 30 minutes, and shearing conditions during the heating may be excluded or obtained in a form less than 500 reciprocal seconds. The pH is in the range of 4-7. The concentration steps are applied by any concentration-techniques known, for example ultrafiltration, ion-exchange, centrifugation, freeze or spray drying etc. The process described here offers the advantage of not requiring complicated apparatus. It is simple to control due to the fact that it does not require high shear conditions or high temperatures and short heating times. It also allows the incorporation of shear-sensitive ingredients and, at the low temperatures used, these do not interact with the protein through Maillard or
other reactions. The temperature thus serves the purpose of pasteurizing the
ingredients. The products formed exhibit good microbiological stability and may be
used to provide a low-calorie food product by replacing at least part of the fat
present, for example, in cheese, yoghurt, margarine, ice cream, salad dressings. It
may also be used for topical application to the body, i.e. in cosmetics.

US patent 4 855 156, Singer et al, 1989
Frozen dessert

This patent includes many illustrations of non-fat and reduced fat whipped frozen
dessert products. Examples of frozen desserts are ice-cream, frozen custards, ice milk,
sherbert, etc. The fat content of these frozen desserts plays a substantial role in
determining the body, texture and flavour characteristics of these products. According
to this patent, non-fat and reduced fat whipped frozen dessert products are provided
wherein the fat is replaced by a proteinaceous macrolloid of denatured protein
particles, preferably whey protein particles. These particles are dependent upon sizes
and shapes in order to derive the desired organoleptic character, and, therefore, the
whey protein has to be subjected to special treatments in order to develop the
particles. The proteinaceous macrolloids are derived from undenatured substantially
soluble proteins, preferably dairy whey. An aqueous protein solution, having a protein
concentration about 10-20% by weight, is subjected to heat treatments in the range
of 80-120°C for 3 seconds up to 15 minutes. A high level of shear is used during the
heat treatment to prevent the formation of large denatured protein aggregates. The pH
is preferably around 6. In the dry state, the particles obtained have a diameter ranging
from about 0.1 microns to about 2.0 microns. They are also spherical. The frozen
whipped desserts comprise, in the end, from 1*10⁹ to 1*10¹² of said denatured protein
particles per cubic centimeter. The patent also discloses examples of both the
preferred methods and procedures for the preparation of macrolloids from whey,
egg white albumen, soy protein and from bovine serum albumin, and of ice cream-
like frozen desserts.
CONCLUSIONS

This review discusses the denaturation and aggregation mechanisms as well as functional properties related to gelation and emulsification, of whey protein and β-lactoglobulin. It deals with applications, in the form of patents, as well as scientific publications.

In recent years, there has developed a desire for low-fat products, and whey protein has proved to be a good replacer of fat. Whey is a cheap dairy product and it has good functional properties. If the mechanisms or factors underlying these properties could be determined, it should be possible to control the relevant processes to improve the character of the whey products. This would remove the present limitations on whey protein materials as food additives and also make it possible to obtain new whey protein products.

The patents described here fall into two classes: one which refers to emulsions and the other to specially sized particles. The section on patents clearly points out that the food industry specially requires more knowledge of the aggregation mechanism in order to increase whey protein utilization.

The section on the scientific publications refers to the results of different investigations. It is clear that little information exist with regard to aggregation mechanisms and that new techniques have to be developed in order to elucidate and control protein aggregation and, by that, particle size. However, this is not impossible as the research in the aggregation mechanism of whey proteins, at the moment, is an explosive area.
REFERENCES


Arnebrant, T., Bäckström, K. and Nylander, T. An ellipsometry study of ionic surfactant adsorption on chromium surfaces in J. Colloid Interface Sci.. submitted.


Harwalkar, V.R., 1979. Comparison of physico-chemical properties of different thermally denatured whey proteins in Milchwissenschaft 34:419.


Hermansson, A.-M., Aggregation and Denaturation Involved in Gel Formation, chapter 5, p. 81-104 in Functionality and Protein Structure, Pour-El, A. (editor), American Chemical Society, Washington, DC, 1979.


161

Zittle, C.A., Della Monica, E.S. Rudd, R.K. and Custer, J.H., 1957. The binding of calcium ions by $\beta$-lactoglobulin both before and after aggregation by heating in the presence of calcium ions in J. American Chemical Society 79:4661-4666.