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Studies on the Binding of Iron and Zinc to Milk Protein Products

Massey University

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

BY

MAYA WULANSARI SUGIARTO

RIDDET CENTRE AND INSTITUTE OF FOOD, NUTRITION AND HUMAN HEALTH

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ABSTRACT

The principal objective of this study was to characterize the binding of iron and zinc to three commercial milk protein products; namely sodium caseinate, whey protein isolate (WPI) and milk protein concentrate (MPC).

The mineral–protein mixtures were prepared by mixing either iron ($FeSO_4.7H_2O$) or zinc ($ZnSO_4.7H_2O$) at a range of concentrations with 1% protein solutions (e.g. sodium caseinate), in 50 mM HEPES buffer at pH 6.6. The mineral–protein mixtures were then centrifuged (10,800 *g*, 20 min) to separate the soluble protein and soluble minerals from the insoluble protein and insoluble minerals. The supernatant, which contained the soluble fractions, was carefully removed and passed through an ultrafiltration membrane to separate "free" minerals from the minerals bound to the soluble proteins.

Under the experimental conditions used in the study, aqueous solutions of ferrous sulphate were relatively insoluble. This was due mainly to the oxidation of ferrous sulphate to the insoluble ferric hydroxide. The addition of a 1% sodium caseinate solution markedly improved the solubility of ferrous sulphate due to the binding of iron to the caseins. The casein molecules were able to bind up to 8 moles Fe/mole protein. Addition of iron above a certain critical concentration (approximately 4 mM) caused the aggregation and precipitation of casein molecules. The loss of solubility was due mainly to the neutralisation of the negative charges on the casein molecules by iron with a consequent decrease in the electrostatic repulsions between the protein molecules.

In contrast to the behaviour of the sodium caseinate, the interactions of iron with the whey protein molecules in WPI did not cause significant precipitation of the iron–WPI mixtures. Whey proteins remained soluble up to a concentration of 20 mM added iron and were able to bind up to approximately 7 moles Fe/mole of protein.

Analysis of the binding curves by Scatchard plots showed that sodium caseinate has a higher binding affinity for iron (log K_{app} = 5.3) than WPI (log K_{app} = 3.6). This confirmed the experimental observation that in sodium caseinate solutions, up to the critical concentration of iron, virtually all iron was bound to the protein molecules whereas in WPI solutions, a small amount of free iron was present. The strong affinity for iron shown by the casein molecules is due mainly to the presence of clustered phosphoserine residues, which are absent in whey proteins.

The binding characteristics of iron to MPC were broadly similar to those for sodium caseinate. However, soluble MPC was able to bind greater amount of iron (45 mg Fe/g protein) than soluble sodium caseinate (20 mg Fe/g protein). In MPC, casein molecules exist in the micellar form and iron was likely to be bound to both the caseins and the colloidal calcium phosphate, probably displacing calcium ions in the process.

The binding properties of proteins were significantly affected by changes in pH. As the pH was decreased from about 6.5 to 5.0, there was a marked decrease in the ability of proteins to bind cations. For example, the amount of iron bound to WPI decreased from approximately 8 to 1 mg Fe/g soluble protein as the pH dropped from 6.5 to 5.0. This decrease was presumably due to the change in the ionisation state of the negatively charged residues. In the case of sodium caseinate and MPC, the situation was complicated by the marked loss of protein solubility at pH values $\leq$ 5.0.

The binding characteristics of zinc to the three milk protein products were broadly similar to those for iron. For sodium caseinate and MPC, there was a critical concentration of added zinc above which proteins lost solubility. Sodium caseinate showed a greater binding affinity for zinc than WPI (Log K_{app} values were 4.8 and 3.3 respectively), while MPC was able to bind more zinc (25 mg Zn/g protein) than sodium caseinate (14 mg Zn/g protein). However, there was one distinctive difference between the binding behaviour of iron and zinc. In the case of WPI, addition of zinc caused precipitation of whey proteins at a concentration above 4 mM added zinc. This was due to the specific binding sites for zinc in the α-lactalbumin fractions.

Oxidation tests, using linoleic acid as the substrate, showed that iron–protein mixtures were able to markedly suppress the rate of oxidation compared to free iron. Among the iron–protein mixtures, iron–sodium caseinate and iron–MPC mixtures suppressed the oxidation rate to a greater extent than iron–WPI mixtures. In iron–sodium caseinate and iron–MPC mixtures, the iron was completely bound to the protein whereas in iron–WPI mixtures, there was still a small amount of unbound iron, which could cause oxidation.

The data obtained from this study will provide valuable information for the production of mineral–protein complexes with good functional properties, which could be used as a source of ingredients in other food products.

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CHAPTER 1
INTRODUCTION

Many populations around the world are deficient in some important minerals. Both iron and zinc are considered essential in the human diet. Iron, as a component of haem in haemoglobulin, myoglobin, cytochromes and other proteins, plays an essential role in transport, storage and utilisation of oxygen. Deficiency of iron, resulting in anaemia, afflicts about 30% of the world's population (Flynn and Cashman, 1997). Zinc is considered to be essential for growth and development, sexual maturation, wound healing and is also involved in the normal functioning of the immune system and other physiological processes. Both iron and zinc are co-factors for a number of enzymes (Flynn and Cashman, 1997). The recommended intakes and hazards of deficiency or excess of these minerals have been established, and a considerable amount of research is being carried out to clarify the role of these minerals in nutrition and human health (Blakeborough *et al*, 1983; Hurrell and Cook, 1990; Hekmat and McMahon, 1998).

Fortification of foods with minerals is a common vehicle for delivering these nutritionally-important minerals in required quantities to the consumer. Many technological problems, however, occur when food products are fortified with minerals, due mainly to the many reactions of minerals with other food components. These problems are reflected as changes in colour, flavour and functional properties of the product. The solubility and chemical reactivity of the added mineral salt determines the kind and extent of reactions that may occur within a food system. The following factors are considered important when choosing a mineral for fortification of foods.

- Relative bioavailability of the mineral.
- Reactivity of the mineral to cause undesired changes in colour, flavour and functionality.
- Stability of the mineral under processing and storage conditions.
- Compatibility with other food components.

Although milk is a good source of iron and zinc, the content of these minerals is too small (0.2–0.4 mg iron/L and 2–6 mg zinc/L) to provide for dietary needs. Hence, addition of iron and zinc to dairy products such as milk or milk protein products could be considered as a potential approach to deliver these minerals to consumers (Gaucheron *et al*, 1997b; Rice and McMahon, 1998). As a result, a number of dairy products (evaporated milk, yoghurt, UHT milk and milk powder) have been fortified with a variety of iron ingredients. However, there are recurrent problems associated with iron fortification, including variable bioavailability, organoleptic defects and product oxidation. For example, when whole milk powder is fortified with iron, catalysis of fat oxidation by iron can lead to an early onset of rancid and oxidised flavours. Formation of sediments, discoloration and taints are often associated with iron fortified dairy foods.

Fortification of infant formulas with zinc is now widely practised, but not much information is available on other dairy-based systems and on the impact of zinc supplementation on product properties.

It is well known that proteins have the ability to bind metal ions. This binding can have a large influence on the chemical reactivity of the metal ion, which consequently is likely to alter the properties of a product. In other words, the bioavailability, organoleptic defects and product oxidation could potentially be modified (improved or made worse) by complexing metal ion with proteins. There is some information in the literature on the iron and zinc binding properties of pure individual milk proteins, but no systematic studies have been carried out on the binding of these minerals by commercial milk protein products, and how they affect the product functionality and bioavailability.

Development of the milk protein–iron and milk protein–zinc complexes could be a novel way of incorporating these minerals into dairy products with high bioavailability and good flavour. In order to allow manufacture of these complexes and thus fortified dairy products, it is essential to have better scientific information on the specific binding of minerals of interest to particular milk proteins.

The overall aim of the project is to generate scientific information on the formation and properties of milk protein–mineral complexes that may allow commercial production of these complexes with good functional properties. The commercial milk protein products used are sodium caseinate, whey protein isolate (WPI) and milk protein concentrate (MPC) because they are structurally and functionally diverse, and industrially available proteins. Iron and zinc are chosen because of their importance in human nutrition and their known deficiencies worldwide.

Specific objectives are as follows:

- To characterize the binding in terms of binding isotherms, binding constants and number of binding sites of iron and zinc to different milk protein products.
- To investigate the effects of solution conditions, e.g. pH and salts on the binding properties of selected protein products in order to identify conditions for optimum binding.
- To investigate the oxidation activity of selected mineral–protein complexes using a model food system.

CHAPTER 2
LITERATURE REVIEW

2.1. Introduction

The focus of this thesis was to study the binding of minerals to milk protein products such as sodium caseinate, whey protein isolate (WPI) and milk protein concentrate (MPC). Because addition of minerals to milk or milk products causes interactions of the minerals with the protein, the following literature review covers the chemistry of the main components of milk with a focus on the interactions of metal binding and the functional properties of the commercial milk protein products. The minerals used in this study are considered due to their essential functions in the human body and their well-known deficiencies in the diet. Therefore, the deficiencies of the iron and zinc are reviewed along with published literature on the metal–protein interactions and the binding study of iron and zinc to milk and milk products. The published literature on how addition of minerals promotes fat oxidation in milk protein products is also covered in this review.

2.2. Milk Proteins

Bovine milk contains approximately 3.5% protein, which falls into 2 principal categories. The first ones are caseins, which constitutes about 80% of the total protein. The other 20% are whey proteins, which remains in solution at pH 4.6 (Walstra and Jenness, 1984). Table 2.1 shows some of the structural and chemical characteristics of the major milk proteins.

Table 2.1. Some structural and chemical characteristics of milk proteins (adapted from Kinsella *et al*, 1989)

Property	Whey			Caseins			
	β-Lg	α-La	BSA	α_{s1}-	α_{s2}-	β-	κ-
Molecular Weight	18362	14194	65000	23612	25228	23980	19005
Total residues	162	123	581	199	207	209	169
Apolar residues [%]	34.6	36	28	36	40	33	33
Isoionic Point	5.2	4.2–4.5	5.3	4.96	5.27	5.2	5.54
Proline residues	8	2	28	17	10	35	20
Phosphoserine groups	0	0	0	8–9	10–13	5	1
Disulphide bonds	2	4	17	0	1	0	1
Thiol groups	1	0	1	0	0	0	0
	Secondary structure (%)						
α-helix	15	26	54	-	-	9	23
β-sheet	50	14	18	-	-	25	31
β-turns	18	-	20	-	-	-	24
Unordered	-	60	-	-	-	66	-
Native conformation	Globular			Extended			

Abbreviation used: β-Lg = β-lactoglobulin; α-La = α-lactalbumin; BSA = bovine serum albumin; α_{s1}-casein; α_{s2}-casein; β-casein; κ-casein

2.2.1. Caseins

The caseins are synthesized in the mammary gland. Compositionally, the hallmark of the caseins is ester-bound phosphate (Ng-Kwai-Hang, 2003). All of the casein polypeptide chains have at least one such group per molecule. All caseins contain sites of phosphorylation with a unique sequence –Ser-X-A-, where X = any amino acid and A = Glu or Ser-P. All of the phosphorylation sites are located in clusters (Wong *et al*, 1996). This clustering of the phosphoserine residues is particularly apparent and this has a marked influence on the metal-binding properties of the caseins with technological and nutritional consequences, e.g. reduced bioavailability of iron and zinc.

Caseins are classified according to their primary structures: α_{s1}-, α_{s2}-, β-, and κ-caseins. The caseins, compared to typical globular proteins that have mainly α-helical and β-sheet structures, contain less secondary and tertiary structures. Most of the secondary structure is likely to be present in the hydrophobic domains. The physical structure and chemical properties of caseins have been extensively reviewed (Swaisgood, 1992; Creamer, 2003; Swaisgood, 2003).

α_{s1}-Casein

The primary structure of α_{s1}-casein contains 199 amino acid residues. It has no cysteine residue and 8 phosphates attached to serines (Ng-Kwai-Hang, 2003). Hydrophobic and charged residues are not distributed uniformly throughout the sequence. There are 3 discernible hydrophobic regions and the unique feature of the primary structure of this calcium-sensitive casein is the clustering of the phosphoseryl residues. The cluster sequence Glu-PSer-X-PSer-PSer-Glu-Glu, where X is Ile or Leu and PSer is phosphoserine, occurs in all known variants of α_{s1}- and β-caseins. These sequences are present in the polar domains of the calcium-sensitive caseins (Swaisgood, 2003).

α_{s1}-Casein can be visualized as a rather loose flexible polypeptide chain. The flexible nature of the polar domain causes the molecular dimensions to be very sensitive to ionic strength and to binding of ions, particularly protons (H^+) and Ca^{2+} (Swaisgood, 1985). It binds about 8 moles Ca^{2+} per mole near pH 7, probably to ester phosphate groups. It aggregates and precipitates at very low concentrations of Ca^{2+} (7 mM Ca^{2+}, 28 mM NaCl) (Walstra and Jenness, 1984). Intermolecular interactions between hydrophobic domains lead to self-association, or association with other caseins (Swaisgood, 1985). Self-association of α_{s1}-casein depends markedly on concentration, pH, ionic strength and kind of ion in the medium, but it is relatively independent of temperature (Walstra and Jennes, 1984).

α_{s2}-Casein

The general characteristics of α_{s1}-casein are shared by the other calcium-sensitive caseins i.e. α_{s2}-casein and β-casein. Structure of α_{s2}-casein is characterized by charged polar domains and hydrophobic domains. The sequences in the polar domains, which may approach a random coil secondary and tertiary structure, are such that clusters of seryl residues are phosphorylated (Swaisgood, 1985). α_{s2}-Casein contains 207 amino acids with 10 prolines, more phosphoserines and more lysines than the other caseins. It has 2 cysteines and 10–13 phosphate groups. Among the caseins, α_{s2}-casein is the least hydrophobic (Ng-Kwai-Hang, 2003).

α_{s2}-Casein has a remarkable dipolar structure with a concentration of negative charges near the N-terminus and positive charges near the C-terminus. It binds Ca strongly and is more sensitive to precipitation by Ca^{2+} than is α_{s1}-casein. It self-associates at neutral pH in the absence of Ca^{2+}, and the association depends markedly on ionic strength and is at a maximum at an ionic strength of about 0.2 (Walstra and Jenness, 1984). Both α_{s1}- and α_{s2}-caseins are precipitated by 6 mM Ca^{2+} at all temperatures (Wong *et al*, 1996).

β-Casein

β-Casein is the most hydrophobic casein and has 209 amino acids sequence. It has no cysteine residues and a high proportion of proline (35 residues), which has a profound effect on its structure (Ng-Kwai-Hang, 2003). β-Casein has a strongly negatively charged N-terminal region (Swaisgood, 2003) while the rest of the molecule is very hydrophobic and has no net charge. Consequently, this molecule is very amphipathic with a polar domain comprising one-tenth of the chains but carrying one-third of the total charge and a hydrophobic domain consisting of the C-terminal three-fourth of the molecule. The outstanding characteristic of the association of β-casein in both the absence and the presence of Ca^{2+} is its strong dependence on temperature (Walstra and Jenness, 1984; Swaisgood, 1985). β-Casein tightly binds about 5 Ca^{2+} per mole, consistent with its ester phosphate content (Walstra and Jenness, 1984).

κ-Casein

κ-Casein constitutes about 10–12% of the whole caseins and consists of 169 amino acids. κ-Casein has 2 cysteine residues and serine residues and is the only protein of the casein family that is glycosylated (Creamer, 2003; Ng-Kwai-Hang, 2003). It has an amphipathic character, which encourages it to form micelles in solution. It plays a crucial role in stabilizing the casein micelles in milk and, after enzymatic cleavage, destabilizing the colloidal casein system.

κ-Casein does not contain clusters of phosphoserine residues in its polar domain as do the calcium-sensitive caseins; hence it does not bind as much Ca^{2+} (1–2 moles Ca^{2+} per mole of protein) and is not sensitive to precipitation by Ca^{2+} (Swaisgood, 1985; Ng-Kwai-Hang, 2003).

2.2.1.1. Calcium binding by caseins

All casein components are able to bind different bivalent metal ions, especially Ca^{2+}. The phosphate groups of the phosphoserine residues constitute the main Ca^{2+} binding sites in the caseins. Hence, the calcium binding capacity follows the order: α_{s2}- > α_{s1}- > β- > κ-casein (Rollema, 1992). Apart from binding to these sites, Ca^{2+} ions also interact with other groups, such as carboxylate groups of aspartate and glutamate residues (Rollema, 1992). Because of the high content of phosphoserine residues, β-, α_{s1}-, α_{s2}-caseins bind polyvalent cations strongly, principally Ca^{2+}, but also Mg^{2+} and Zn^{2+} (Walstra and Jenness, 1984; Rollema, 1992, Wong *et al*, 1996).

Binding of calcium to the caseins at neutral pH reduces their negative charge, which diminishes the electrostatic repulsion between the molecules. In this way, calcium binding promotes hydrophobic interactions which can lead to increasing association and ultimately to precipitation. The solubility of α_{s2}-, α_{s1}- and β- caseins is strongly affected by the binding of calcium ions. Upon addition of calcium these proteins remain soluble until a critical amount of calcium is bound, after which an additional

increase in calcium ion concentration leads to a sharp decrease in their solubility. For κ-casein, such effects are not observed because of its low phosphoserine content (Rollema, 1992).

2.2.1.2. Casein micelles

Almost all casein in milk exists in coarse spherical particles known as micelles, ranging in size from about 30–300 nm and comprising of 20–150,000 casein molecules (Schmidt, 1980; Walstra and Jenness, 1984; Swaisgood, 1985). Typically, micelles are composed of 92% protein, with α_{s1}-, α_{s2}-, β- and κ-casein in a ratio of 3:1:3:1, and 8% inorganic matter, composed primarily of calcium (2.87%) and phosphate (2.89%), citrate (0.4%), and small amount of magnesium, sodium and potassium (Schmidt, 1980; Walstra and Jenness, 1984; Rollema, 1992). The micelles are very open and largely hydrated containing about 2–4 g H_2O per g protein, depending on the method of measurement (De Kruif and Holt, 2003).

The structure of casein micelles has received a lot of attention and has been reviewed extensively (Rollema, 1992, Holt and Horne, 1996; Dalgleish, 1997; De Kruif and Holt, 2003; Horne, 2003). The earlier model of casein micelles was proposed by Waugh (1971) called the coat-core model. This model considered the core of the micelle to consist of spherical particles formed by α_s- and β-Caseins, whereas the coat consisted of κ-casein. In the review by Rollema (1992), another idea concerning casein micelle structure was mentioned called the internal structure model, which specifies the mode of aggregation of the different caseins.

The model, which has received a lot of attention in recent years, is the submicelle model (Schmidt, 1982; Walstra and Jenness, 1984; Walstra, 1990). The submicelle model assumes that the micelle is composed of discrete subunits called submicelles. Submicelles, like globular proteins, have a dense hydrophobic core, in which are tucked most of the hydrophobic parts of the casein molecule and a less dense, hydrophillic outer layer, containing most of the acidic (carboxylic and phosphoric) and some of the

basic group. The submicelles are linked together in the micelles by colloidal calcium phosphate (CCP) and hydrophobic bonds, with contributions from other secondary forces such as hydrogen bond. The presence of κ-casein plays an important role for the stability of casein micelle suspension. The κ-casein macropeptides (which is hydrophilic) protrude into solution, interacting with water, forming a layer composed of highly negatively charge protein segments. This provides electrostatic and steric repulsion against further aggregation of the micelles (Figure 2.1).

More recently, submicelle models have been challenged on the basis that they do not explain changes in the micelle resulting from calcium sequestration, heating and subsequent cooling of milk and lowering pH of skim milk.

A model for the casein micelle was proposed by Holt (Holt, 1992). Holt model proposes that the regions of high scattering power are calcium phosphate nanoclusters, small crystallites that interact with the phosphoserine clusters of the casein molecules. Because some of the individual caseins, α_{s1}- and α_{s2}-caseins, have more than one such grouping, crosslinking and network formation is possible to give a microgel particle. Unfortunately, there is no inherent mechanism in this model for limiting gel growth. The model also has no role for κ-casein. κ-Casein has no phosphate cluster to bind with the nanoclusters. The Holt model does not explain the observed surface location of κ-casein in the natural micelle.

A model proposed by Horne (1998) assumes that a balance of electrostatic (through bridging across calcium phosphate nanoclusters) and hydrophobic interactions between casein molecules control the assembly of the casein micelle. Two or more hydrophobic regions from different molecules within the caseins form a bonded cluster. Growth of these polymers is inhibited by the protein charged residues whose repulsion pushes up the interaction free energy. Neutralisation of the phosphoserine clusters by incorporation into the colloidal calcium phosphate diminishes that free energy as well as producing the second type of cross-linking bridges. κ-Casein acts as a terminator for both types of growth, because it contains no phosphoserine cluster linkage via CCP and

no other hydrophobic anchor point to extend the chain. This model is described graphically in Figure 2.2.

A major failing of the earlier micelle models was their lack of a plausible mechanism for assembly, growth and, more importantly, termination of growth. All such elements are in place in the dual binding model (Horne, 1998). Furthermore, the concept of a localized excess of hydrophobic attraction over electrostatic repulsion as well as permitting the visualization of micellar growth successfully accommodates the response of the micelles to changes in pH, temperature, urea addition or removal of calcium phosphate by sequestrants (Horne, 2003).

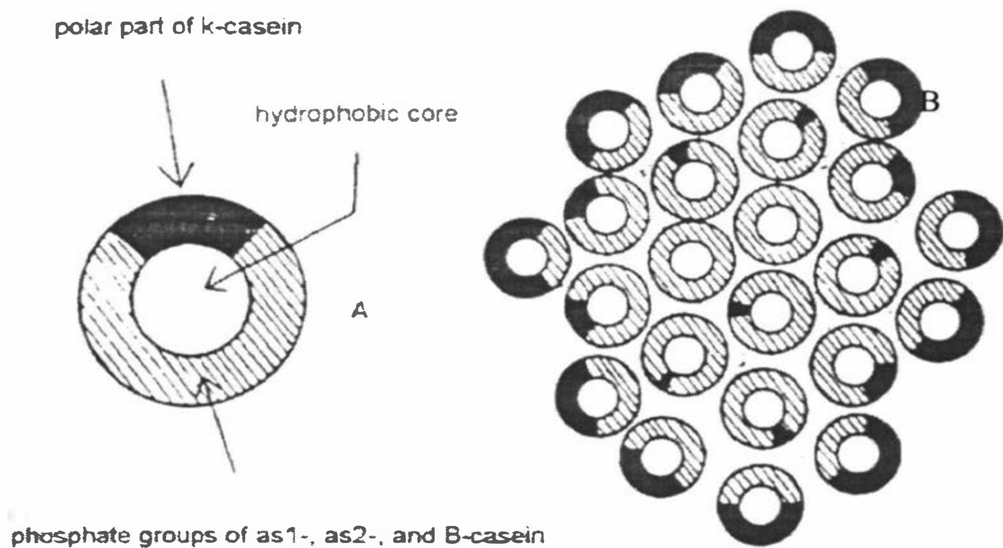


Figure 2.1. Casein submicelle model according to Schmidt (1982): A. submicelle; B. casein micelle.

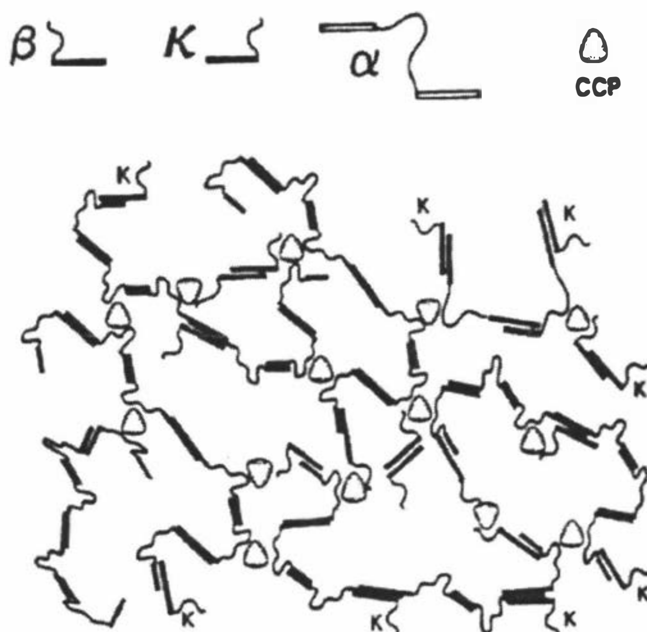


Figure 2.2. Dual bonding model of casein micelle structure (Horne, 1998).

2.2.2. Whey Proteins

The term whey proteins refer to milk proteins remaining in the serum (or whey) after precipitation of the caseins from milk at pH 4.6 at 20°C. This fraction accounts for approximately 20% of total protein (Ng-Kwai-Hang, 2003).

Unlike caseins, whey proteins are globular and have more organized secondary and tertiary structures (Ng-Kwai-Hang, 2003). They exist as discrete molecules with varying numbers of disulfide crosslinks. Compared to caseins, these proteins are more heat sensitive, less sensitive to calcium, can engage in thiol disulfide interchange, and form disulfide-linked dimers or polymers, e.g. with κ-casein (Kinsella, 1984).

There are four major proteins, denoted as β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins. These account for > 95% of the non-casein proteins. Both β-lg and α-la are synthesized in the mammary gland whereas serum albumin is

transported to the mammary gland via the blood serum (Ng-Kwai-Hang, 2003). Published literature on major whey proteins have been reviewed by Swaisgood (1982), Hambling *et al* (1992), Brew (2003), Creamer and Sawyer (2003), Sawyer (2003) and Zhang and Brew (2003).

β-Lactoglobulin (β-Lg)

β-Lg accounts for more than 50% of the total whey protein in bovine milk and about 10% of the total milk protein in cow's milk (Creamer and Sawyer, 2003). It has a monomeric molecular weight of 18,300 Da (Kinsella, 1984; Walstra and Jenness, 1984). The primary structure of β-Lg consists of 162 amino acids. It has 5 cysteine residues capable of forming disulphide bonds between residues 66–160, 119–121 or 106–119 (Ng-Kwai-Hang, 2003). It has a single free thiol (R–SH) group which is of great importance for changes occurring in milk during heating since it facilitates thiol/disulphide (R–SH/S–S) interchange reactions which allow the formation of new structures or intermolecular disulfide-bonded dimers and polymers (Kinsella, 1984). It is also involved in reactions with other proteins, notably κ-casein and α-La upon heating (Walstra and Jenness, 1984). Up to now, there are 13 known genetic variants of β-Lg: A, B, C, D, E, F, G, H, I, J, W, Dr and K^B (Sawyer, 2003). The A and B genetic variants are the most common and exist in almost the same frequency.

In milk, native β-Lg occurs as dimer at pH 3–7. Under conditions close to physiological, β-Lg forms dimer reversibly and that dimerisation is dependent on ionic strength, pH, temperature and protein concentration (Creamer and Sawyer, 2003). The dimer is stable between pH 5.5 and pH 7.5, but dissociates due to strong electrostatic repulsions below pH 3.5. Between pH 3.5 and pH 5.2, the dimers form octamers. Above pH 7.0, β-Lg undergoes reversible conformational changes, and above pH 8.0, it is unstable and forms aggregates of denatured proteins (Lyster, 1972).

α-Lactalbumin (α-La)

α-La is the second most abundant of the whey proteins accounting for 20% of them (2 to 5% of the skim milk total protein) (Kinsella, 1984; Wong *et al*, 1996). Like the other whey proteins, α-La is a compact globular protein and has a molecular weight of 14,175 Da (Ng-Kwai-Hang, 2003). It has a sequence of 123 amino acids with 8 cysteine residues, which are connected by 4 disulphide bridges between positions 6–120, 28–111, 61–77 and 73–91. There are four inter-chain disulphide bonds, but no sulphydryl groups are present in α-La. These bonds do not polymerise upon heat treatment hence α-La is relatively heat stable (Kinsella, 1984).

α-La plays a very important role in the biosynthesis of lactose by being the modifier in the lactose synthetase complex. Several studies indicate that α-La binds calcium, phospholipids and membranes (Zhang and Brew, 2003).

All known α-La contain a tightly bound Ca^{2+} that strongly influences stability and structure but is not required for their activity in lactose biosynthesis. At low pH, the Ca^{2+} is removed and results in a large reduction in protein stability. At room temperature and low ionic strength, the apoprotein (calcium-free form) undergoes a change in three-dimensional structure to a state where the protein retains most of its native secondary structure but has no fixed tertiary structure, know as 'molten globule' state (Zhang and Brew, 2003).

Besides Ca^{2+}, other metal ions, including Zn^{2+}, Mn^{2+}, Hg^{2+}, Pb^{2+} and lanthanides bind to α-La and the binding sites for calcium, zinc and for a second weakly binding Ca^{2+} have been structurally characterized by X-ray crystallography (Zhang and Brew, 2003).

Bovine serum albumin (BSA)

BSA is identical to the serum albumin found in the blood. It is a single polypeptide of 582 amino acid residues with a calculated molecular weight of 66,433 Da. It is synthesized in the liver and gains entrance to milk through the secretory cells and

accounts for about 5% of the total whey proteins (Walstra and Jenness, 1984; Haggarty, 2003). BSA has one free thiol (Cys 34) and 17 disulphide linkages, which hold the protein in a structure consisting of nine loops. The molecule is considered to have three major domains, each consisting of two large double loops and a small double loop, and assumes an ellipsoidal shape. The N-terminal region is more compact than the C-terminal region (Ng-Kwai-Hang, 2003).

Immunoglobulins

Immunoglobulins are the largest (molecular mass > 1000 kDa) and most heterogenous of the major whey proteins. They are antibodies synthesized in response to stimulation by macromolecular antigens foreign to the animal. Five types of immunoglobulins have been classified in bovine milk; IgG, IgA, IgM, IgE and IgD. They account for up to 10% of the whey proteins and exist as monomers or polymers of two kinds of polypeptide chains, light (L) (MW 22,400 Da) and heavy (H) (MW of 50–60,000 Da), each containing about 200 and 450–600 amino acid residues, respectively. The light and heavy chains (two of each) are joined by disulphide linkages to form the basic immunoglobulin structure.

Other whey proteins

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

2.3. Milk Protein Products

2.3.1. Caseinate

Four types of casein products are available: acid caseins, rennet caseins, caseinates, and co-precipitates (Southward, 2003). Acid caseins are simply isoelectric precipitates (pH 4.6) and they are not very soluble. Likewise, rennet casein, which is produced by treatment with chymosin (rennet), is not very soluble, especially in the present of Ca^{2+}, because the polar domain of κ-casein has been removed.

Caseinates are produced from skim milk by adding acid (HCl or lactic acid) or microbial cultures to precipitate the casein from the whey at pH 4.6. The precipitated casein is then washed with water and then dried. Such a product is insoluble in water. The acid–precipitated casein can then be resolubilised with alkali or alkaline salt (using calcium, sodium, potassium, or magnesium hydroxide) (to about pH 6.7) and spray dried to form caseinate (Walstra and Jenness, 1984; Mulvihill, 1992; Wong *et al*, 1996). The dried powder dissolves completely in water to produce a viscous, sticky, straw-coloured solution (Southward, 2003). Figure 2.3 outlines briefly the manufacture of sodium caseinate.

The acid and alkaline treatments disrupt the native structure of the casein particles and caseinates have no similarity to the native casein micelles in milk (Morr, 1982; Kinsella, 1984). The alkaline treatment solubilises the caseins by altering the net charge which overcomes hydrophobic interactions (Kinsella, 1984). The resulting protein system consists of polymerized casein subunits that are probably arranged in an ordered structure which allows maximum interaction through hydrophobic bonding and also retains the polar, acidic groups in an exposed position where they can be readily influenced by pH and ionic composition of the medium (Morr, 1982).

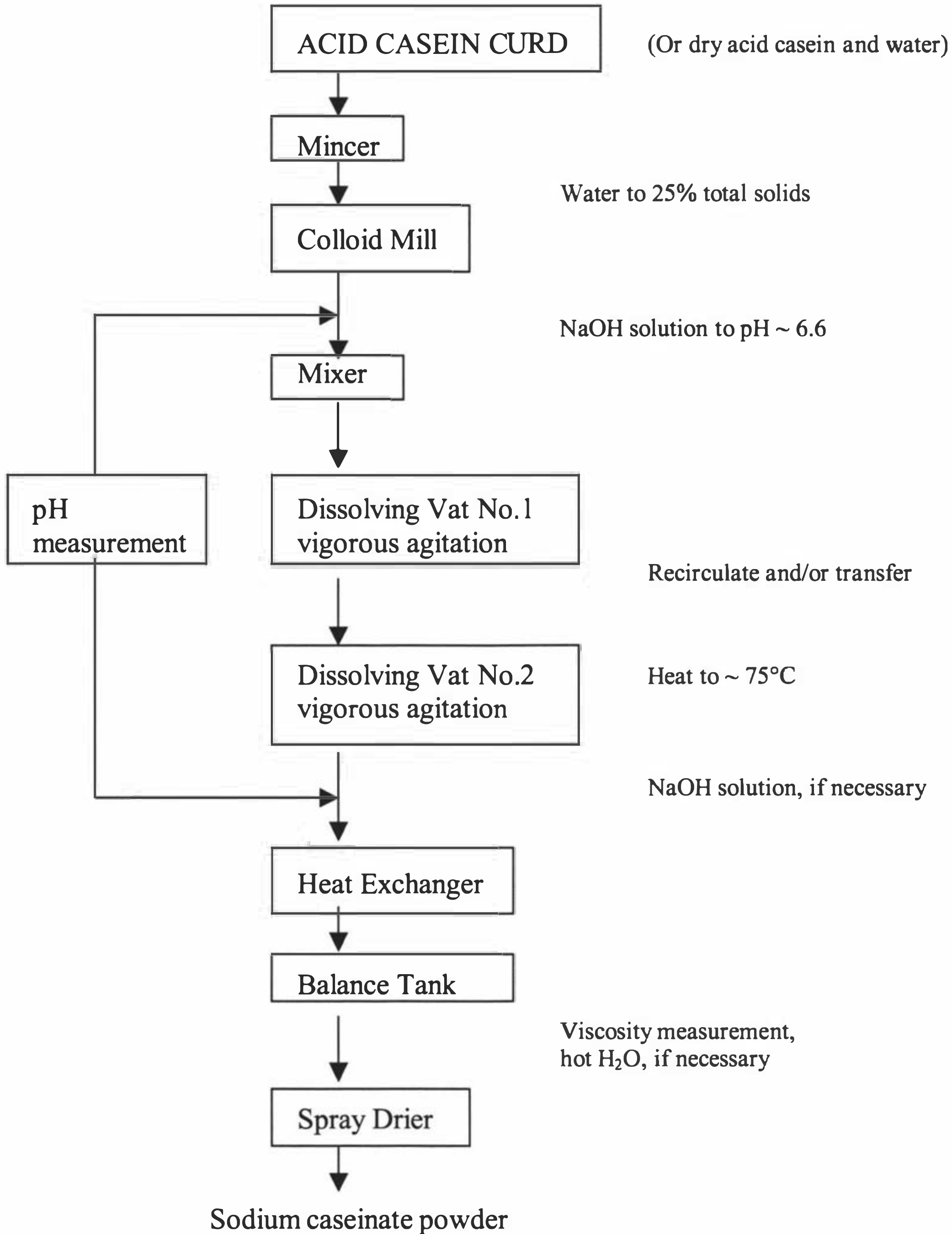


Figure 2.3. Sodium caseinate manufacture (from Mulvihill, 1992).

Sodium caseinate is highly water-soluble casein and can be dispersed rapidly in aqueous mixtures (Kinsella, 1984). In general, commercial sodium or potassium caseinates are more soluble and possess better functional properties than the calcium derivatives. This is attributed to the larger size and more strongly interacting calcium caseinate aggregates whose size are attributed to ionic cross-linking by calcium. Caseinate aggregates are smaller and more sensitive to pH and ionic strength than the colloidal phosphate-containing casein micelles in milk (Kinsella, 1984).

Solubility of caseinates strongly depends on pH, salt content and type of cation. Caseinates are completely soluble at pH > 5.5. Minimum solubility was observed around pH 4 to 4.5. Above pH 4.5, solubility increased to 90% (Kinsella, 1984). Some perturbation of the solvent properties such as addition of NaCl or a water miscible organic solvent (ethanol or acetone) can also cause casein precipitation (Vojdani, 1996).

2.3.2. Whey Protein Concentrates (WPC) and Whey Protein Isolate (WPI)

WPC is a more concentrated form of whey components. Ultrafiltration and diafiltration membrane technology is used to further concentrate and separate the protein and fat (retentate) from the lactose and minerals (permeate). The whey is then dried to obtain WPC, which is highly soluble, with protein levels ranging from 34% to 90% (Walstra and Jenness, 1984; Burrington, 1998).

WPI are products of whey component with a protein content of over 90% (Foegeding and Luck, 2003). Ion exchange chromatography is one of the methods used in the manufacture of whey protein isolates. Two major ion exchange fractionation processes have been commercialized for the manufacture of WPI (Figure 2.4). The processes also remove lactose and minerals which tend to reduce protein functionality due to complex formation and protein–ion interactions. A review of these processes is described in Mulvihill (1992).

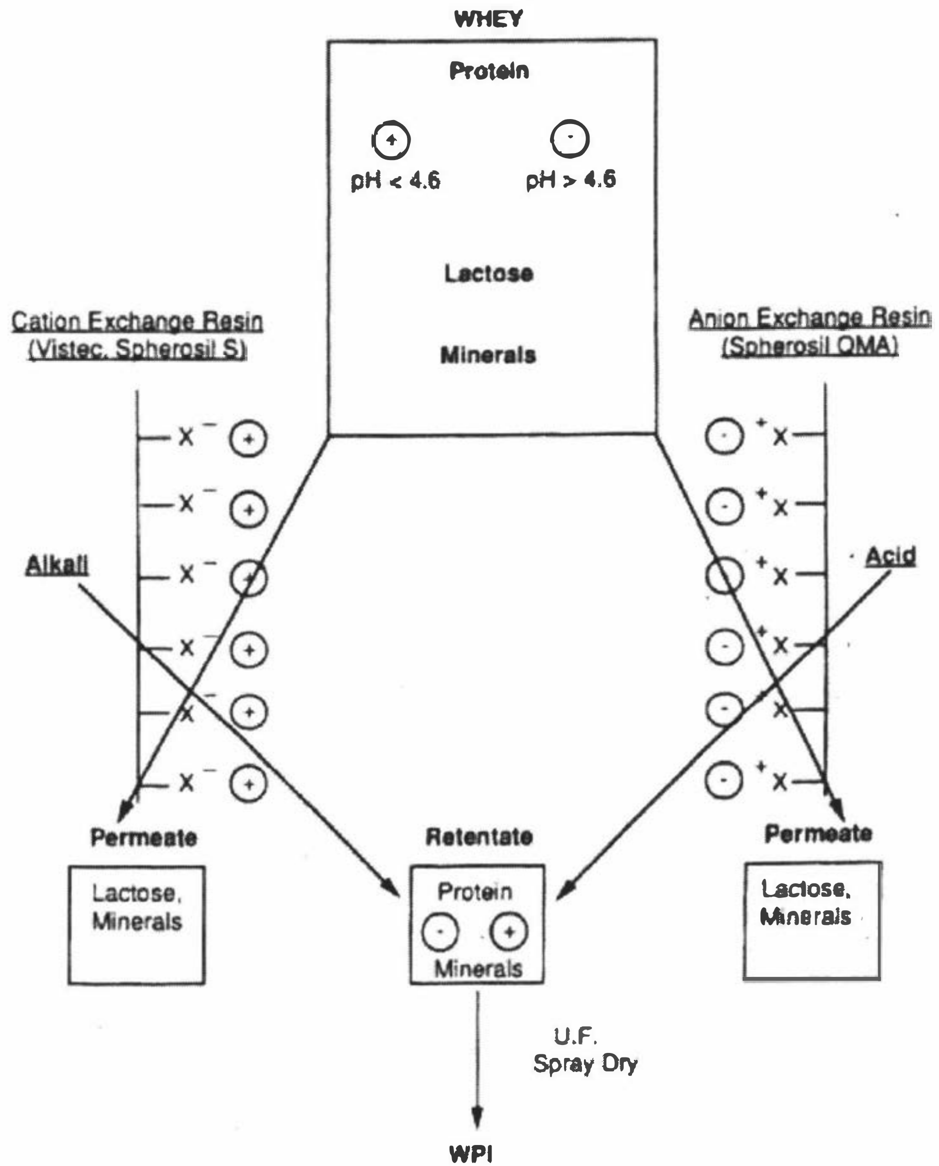


Figure 2.4. Production of WPI by ion exchange adsorption (from Mulvihill, 1992).

The proteins in these products are not denatured, and their functional properties are largely retained (Wong *et al*, 1996). WPI has a wide range of food ingredient and industrial applications and because of its high protein content, WPI functions as water-binding, gelling, emulsifying and foaming agents (Foegeding and Luck, 2003).

Whey proteins are unique among the milk proteins since in their native conformation they are soluble at low ionic strength over the entire pH range required for food application. However, being globular proteins, their solubility decreases at high salt concentrations due to salting out and they are susceptible to thermal denaturation at temperatures > ~70°C (Mulvihill, 1992). Solubility at pH 4.6 is widely used as an index of the extent of denaturation caused by processing and storage of protein-rich whey products. Processing treatments used in the manufacture of WPC and WPI may sometimes cause small amounts of denaturation, which tends to reduce their solubility. The solubility of whey proteins is generally not altered by ultrafiltration or spray drying, although pasteurization may cause up to 20% denaturation with a consequent loss of solubility (Singh, 2003).

2.3.3. Milk Protein Concentrate (MPC)

Milk protein concentrates are processed directly from skim milk by ultrafiltration/diafiltration (Mulvihill, 1992). MPC contains a range of protein content from 56% to 82% protein (Mulvihill, 1992; Burrington, 1998). The casein in MPC is in a similar micellar form to that found in milk while the whey proteins are also reported to be in their native form (Mulvihill, 1992).

A general process flow diagram for the manufacture of MPC is shown in Figure 2.5. MPC of 50–90% protein content (dry basis) can be produced by this process with the original casein/whey protein ratio, depending on the ultrafiltration and diafiltration parameters chosen. Through ultrafiltration/diafiltration process, the feed solution is pumped under pressure over the surface of a suitable membrane. The retentate stream will be enriched in the retained macromolecules, while the permeate stream will be

depleted of the macromolecules. By manipulating the membrane pore size and pressure drop over the membrane, a controlled separation of particles from the fluid can be achieved in parallel with concentration. During the ultrafiltration and diafiltration stages of MPC 85 manufacture, the salt and protein concentration of the permeate and retentate changes as skim milk is transformed into MPC 85 concentrate.

MPC is not very soluble and its solubility is improved with increase in temperature. The solubility of MPC 80 was investigated by Zwijgers (1992) who reported that MPC 80 is hardly soluble at 16°C (> 40 g sedimentible material/100 g solution). The solubility improves a little at temperatures 40–60°C (< 0.5 g sedimentible material/100 g solution). Pierre *et al* (1992) observed that raising the reconstitution temperature to 50°C instead of 24°C increases the solubility index from 74 to 97.2%.

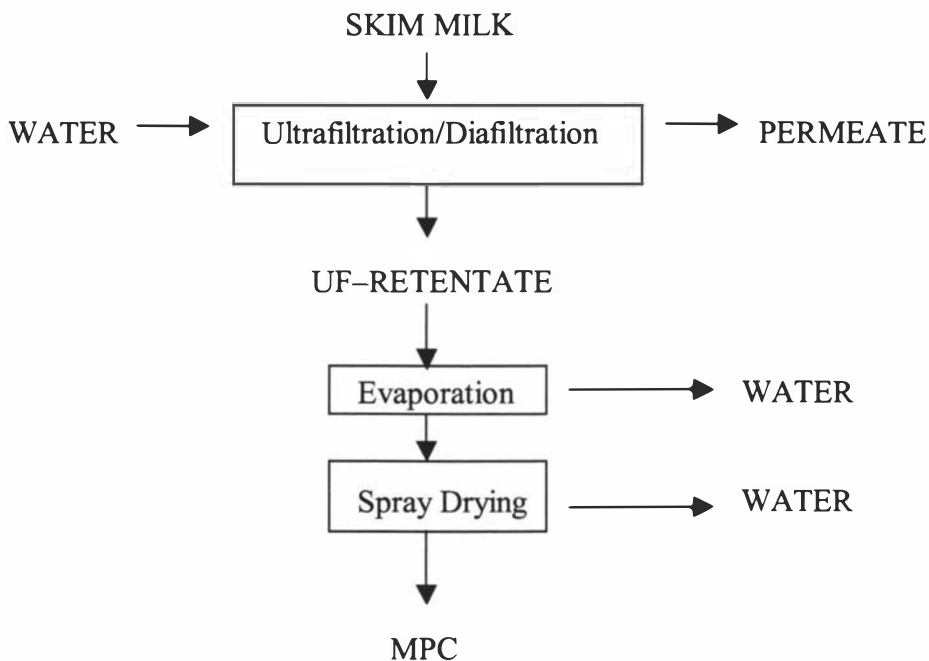


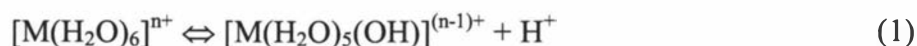
Figure 2.5. Schematic diagram of the MPC manufacturing process (from Novak, 1991)

2.4. Metal–Protein Interactions

Proteins are known to have the ability to bind metal ions. The metal-protein interactions, which occur during the binding, depend not only on the type of metal and the available sites in the protein, but also on the environmental conditions. Important aspects of metal-protein interactions are summarized below (more details are given in Gillard and Laurie (1988)).

2.4.1. Properties of Metal Ions

Metal ions are most familiar as their salts, and in nearly all dietary intakes, will at one point or another be present as an aqueous solution containing the solvated (aquated) metal ion. The common ions of diet and metabolism are, within physiological and dietary ranges of pH, not subject to much hydrolysis. However, if the pH of a solution containing ions, such as iron (III), zinc (II), or additives like bismuth or titanium rises much above 3.5, then the formation of solid oxo- and hydroxo- containing species might be expected.



$$[M(H_2O)_6]^{n+} \Leftrightarrow [M(H_2O)_5(OH)]^{(n-1)+} + H^+ \quad (1)$$

However, the properties of metal ions most relevant to their interactions with protein and their formation of complex compounds with the protein are due to their interaction with their environment. This complex formation is represented, in general terms, with a protonic ligand, H_xL, (which is here a protein) in the equation:

$$H_xL + M^{x+} \Leftrightarrow ML + xH^+ \quad (2)$$

There are a few things to note regarding this equilibrium (equation 2) as discussed briefly below.

1. Stabilities

Most metal ions, in their interactions with proteins, form complexes quite rapidly. Their equilibria are established quickly, so that thermodynamic treatments are sensible.

General rules for stability of metal complexes are:

- More highly charged ions will bind more strongly than less highly charged ions. (The greater the stability (or equilibrium) constant, the more stable the complex).

- If there is no change of coordination environment (that is if the number of binding sites for the metal ion and their relative distribution and space remain the same) within a like-charged series, the smaller the ion, the more stable the complex. Example: $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} < Zn^{2+}$.

The pH will be of importance, because nearly all aqueous equilibria between metal ions and proteins will involve competition of metal ions with protons for binding sites. The distribution of metal ions between the aquo-species and protein-complex species will be dependent upon pH, and in acid regions (e.g. in stomach), much less metal ion will be complexed.

2. Rates (Kinetic)

The rates at which the metal ions come to equilibrium vary with the metal ion and its oxidation state. Labile systems will come into equilibrium in seconds, whereas inert systems taking longer than a minute to come to equilibrium.

3. Oxidation and reduction

Some metals, especially the transition elements, have more than one oxidation states. Metal ion can be more stable in one oxidation state than the other. Hence, stabilization of oxidation states of transition metal by protein environment is important.

4. Classification of metal ions as Lewis acids

The possible binding sites for metal ions on proteins range from the peptide group itself, to terminal carboxylate, terminal amino groups, side chain substituents like thiol and thiolate, or donors in aromatic constituents of side chains. Of all these possible binding sites, the nature of the metal ion will clearly determine the likely site for its binding.

Some metal ions prefer sulphur-sites, whereas others would have a much greater affinity for oxygen-containing sites.

2.5. Binding of Metal Ions to Proteins

Metal–protein complexes play a major role in the absorption of the metal. Hence it is necessary to determine the strength and the number of binding sites and the magnitude of the interaction between multiple binding sites to understand the biochemical systems. Multiple binding sites may be independent of one another or interactive, in which case occupation of one site influences the binding at the second site. Independent binding sites may be involved in tight binding or weak binding. When a molecule binds to a proteins that has many weak binding sites, either the affinity of all the binding sites changes abruptly to a new value or the affinity of one or more of the sites changes gradually. In the second case, the affinity continues to change as more ligand molecules bind. The affinity can either increase (positive cooperativity) or decrease (negative cooperativity). When the binding of the first molecule facilitates subsequent binding, the situation is called positive cooperativity. Negative cooperativity occurs when a bound ligand reduces the binding of subsequent ligand molecules, as often observed in ion binding to proteins (Dahlquist, 1978; Regenstein and Regenstein, 1984).

2.5.1. Determining Binding Sites and Binding Constants

The most commonly used method for interpreting binding data is Scatchard equation (Dahlquist, 1978; Regenstein and Regenstein, 1984; O'Neill, 1996). This model assumes the presence of a number of protein molecule, **P**, in solution, each possessing **n** indistinguishable and independent binding sites. The equilibrium between a ligand, **A** and a protein with one binding site may be expressed as (O'Neill, 1996):

$$PA_{(n-1)} + A \Leftrightarrow PA_n \quad (1)$$

The association constant, K, is defined as:

$$K = \frac{Products}{Reactants} = \frac{[PA]}{[P][A]} \quad (2)$$

So

$$[PA] = K\,[P]\,[A] \tag{3}$$

Since $P_{(total)} = [PA] + [P]$,

$$[PA] = K\,[A]\,[P_{(total)} - [PA]] \tag{4}$$

Or

$$\frac{[PA]}{[P(total)]} = \frac{K[A]}{1+K[A]} \tag{5}$$

Now,

$$\frac{[PA]}{[P(total)]} = v \tag{6}$$

where v is the number of moles of ligand bound per mole of total protein. Thus,

$$v = \frac{K[A]}{1+K[A]} \tag{7}$$

Or

$$\frac{v}{A} = Kn - Kv \tag{8}$$

Plotting v/A versus v gives the Scatchard plot, which has a slope of $-K$, and y-intercept nK. An advantage of using this method of analysis is that if the binding sites are not both identical and independent, the plot will not give a straight line. Another advantage of Scatchard equation is that, the value of n can be read directly from the graph.

For binding site heterogeneity, the Scatchard plot appears to be biphastic with different limiting slopes at low and high fractional saturation (Dahlquist, 1978). Since the slope of the Scatchard plot for a system showing a single affinity is simply the negative of the association constant, it might seem reasonable that the limiting slopes represent the apparent affinity at low and high degrees of saturation. An apparent association constant K_{app} can be defined, providing the range in affinities is not too great. Thus, extrapolation of the intercept to zero saturation gives the average formation constant for binding, while the limiting slope at high saturation gives the average dissociation

constant (K_{diss}) for the interactions of the various binding sites with ligand (Dahlquist, 1978).

Equation (8) may be rearranged to give

$$\frac{1}{v} = \frac{1}{nK[A]} + \frac{1}{n} \qquad (9)$$

Plotting $1/v$ versus 1/A gives rise to the Klotz, or double reciprocal plot, which has a slope of $1/nK$ and intercept 1/n.

2.6. Iron and Zinc Deficiencies

Iron and zinc are amongst the essential minerals needed in the human body and they play crucial roles in the functions of human body. As a component of haem in haemoglobin, myoglobin, cytochromes and other proteins, iron plays an essential role in the transport, storage and utilization of oxygen. It is also a co-factor for a number of enzymes (Flynn and Cashman, 1997). Zinc is essential for growth and development, sexual maturation and wound healing, and it may also be involved in the normal functioning of the immune system and other physiological processes. It is a component of the hormone, insulin, and aids in the action of a number of hormones involved in reproduction, as well as being required for the synthesis of DNA, RNA, and proteins and as a co-factor for many enzymes involved in most major metabolic processes (Flynn and Cashman, 1997).

Deficiencies of iron and zinc are quite widespread and affect many populations around the world. Deficiencies of these minerals can result in many health problems. Iron deficiency results in anemia, and has been recognized as endemic in women of childbearing years, in adolescents, and in children (Hekmat and McMahon, 1998; Rice and McMahon, 1998). In children, it can retard psychomotor development and mental performance. In pregnant women, it can result in premature delivery as well as foetal and maternal mortality. In addition, iron deficiency can reduce work capacity, impede

body temperature regulation, impair immune response and perhaps lead to a higher incidence of infection (Hurrell and Cook, 1990). The major consequences of zinc deficiency have been shown to be growth retardation, retarded sexual maturation, skin lesions and iron deficiency anemia (Harzer and Kauer, 1982; Blakeborough *et al*, 1983; Hurrell and Cook, 1990; Flynn and Cashman, 1997).

Although milk is a good source of these minerals, its iron content (0.2–0.4 mg/L) and zinc content (2–6 mg/L) is too small to provide for dietary needs (Flynn and Cashman, 1997). Hence, fortification of foods with these minerals could be considered as a potential approach to prevent the deficiencies caused by iron and zinc. One way of delivering these minerals to consumers is through adding iron and zinc to dairy products, such as milk or milk proteins products.

2.7. Iron Binding Studies

2.7.1. Iron in Bovine and Human Milk

In general, milk and milk products are very poor sources of iron, and cow's milk contributes little to total iron intake. This area has been reviewed by Flynn and Cashman (1997). Mean iron concentration in mature human milk (0.3 mg/L) is found to be lower than that of cow's milk (0.5 mg/L). The iron content of human milk decreases with advancing lactation and decreases in mature milk.

The distribution of iron in both cow's and human milk was described by Flynn and Cashman (1997). In cow's milk, 14% of the iron occurs in milk fat where it is associated with the fat globule membrane. About 24% of the iron is bound to casein, while 29% is bound to whey proteins and 32% is associated with a low-molecular weight fraction. In human milk, 33% (16–46%) of the iron is associated with the lipid fraction and there is evidence that it is bound to xanthine oxidase, an enzyme that is a component of the fat globule membrane. A significant portion (~ 32%, range 18–56%) of the iron is associated with a low molecular weight fraction (< 15,000 Da), which has

not yet been characterized. About 26% is bound to whey proteins, probably mainly to lactoferrin, which is present in mature human milk at a concentration of 1.6–2 g/L, and is only 3–5% saturated with iron.

2.7.2. Sources of Added Iron to Milk or Milk Products

Studies have been done by many researchers to investigate the 'optimum' binding of iron to milk proteins. Different iron sources, milk sources and conditions have been used in these studies. The iron was added either directly into the milk or to the components of the milk. These will be discussed below.

Sources of iron

There are different types of iron sources that can be used as added iron to form iron-protein complexes. They can be classified as: water-soluble, poorly water soluble (but readily soluble in dilute acids), water-insoluble (and poorly soluble in dilute acids) and experimental compounds (Hurrell and Cook, 1990). Some examples are given in Table 2.2.

The most common iron compound used in the study of iron binding is the iron salt. Some of these salts, which have two oxidation states (Fe^{2+} and Fe^{3+}), are completely dissolved in water and in milk. However, they have the disadvantage of freely interacting with the constitutive elements of milk, which may alter its sensory properties (Gaucheron, 2000). The ferrous form of iron mostly used was as ferrous sulfate ($FeSO_4$) (Hegenauer *et al*, 1979a; Nelson and Potter, 1979) and ferrous chloride ($FeCl_2$) (Gaucheron *et al*, 1996, 1997b). The ferric iron used was normally ferric chloride ($FeCl_3$) (Demott and Dincer, 1976; Gaucheron *et al*, 1997a, 1997b) and also ferric pyrophosphate ($Fe_4(P_2O_7)_3$) (Nelson and Potter, 1979). Some chelated forms of iron used were ferric-ethylenediaminetetraacetate (Fe-EDTA) and ferric-nitrilotriacetate (Fe-NTA) (Carmichael, 1975; Hegenauer *et al*, 1979a). Other forms of iron that have been used were ferric lactobionate and ferric fructose (Hegenauer *et al*, 1979a).

Elemental iron has also been used. This is obtained by reduction under H_2 or CO by electrolysis or by the carbonyl process. These compounds were powders with various particle sizes, poorly soluble or insoluble in water and chemically inert. They have the disadvantage of being used only in solid dehydrated food because they do not dissolve in neutral liquids. Examples of these iron are carbonyl iron and electrolytic iron (Gaucheron, 2000).

Iron complexed to proteins or phosphopeptides such as iron–whey proteins, iron–caseinate, iron–protein succinylate and iron–phosphopeptide were amongst the iron sources which have been used in fortifying dairy products with iron (Gaucheron, 2000). These iron complexes have iron binding sites of mainly amino acids such as PhosphoSer, Asp and Glu. The iron bound to these compounds is generally not exchanged and does not react with components of milk (Gaucheron, 2000).

Sources of milk proteins

The iron has been added either directly to whole or skim milk or to individual purified milk proteins. Table 2.3 shows the summary of different protein sources used in the iron-binding study.

Table 2.2. Major iron compounds used in food fortification (from Hurrell and Cook, 1990).

Iron Compound	Relative Bioavalability [a]		Relative Cost [b]
	Rat	Man	
Water Soluble			
Ferrous Sulphate	100	100	1
Ferrous Gluconate	97	89	5
Ferric ammonium citrate	107	NA	5
Ferrous ammonium sulphate	99	NA	2
Poorly Water Soluble			
Ferrous succinate	119	92	4
Ferrous fumarate	95	100	1
Ferric saccharate	92	75	4
Water Insoluble			
Ferric orthophosphate	6-46	31	4
Ferric pyrophosphate	45	39	4
Elemental Iron	8-76	13-90	1
Experimental			
Sodium iron EDTA	*	*	10
Bovine haemoglobin	NA	NA	NA

[a] Bioavalability is expressed as a percentage relative to that of $FeSO_4.7H_2O$

[b] Relative to the cost of $FeSO_4.7H_2O$

* Depends on the food vehicle

NA Data not available

Table 2.3. Summary of different protein sources used in various iron binding studies

LITERATURE	SOURCES OF PROTEIN	Comments
Carmichael *et al*, 1975	Pasteurised skim milk	Casein was obtained from acid precipitation
Demott and Dincer, 1976	Raw milk	Milk was chilled to 4°C
Manson and Cannon, 1978	α_{s1}-casein B, β-casein A', β-casein A' phosphopeptides	
Hegenauer *et al*, 1979a	Raw milk, homogenized (pasteurized) milk, and skim milk (pasteurized)	Raw milk was chilled to 4°C
Nelson and Potter, 1979	Industrial casein (Land O'Lakes casein)	
Nagasako *et al*, 1993	Lactoferrin	Isolated from sweet cheese whey
Baumy and Brule, 1988b	β-casein	Isolated from sodium caseinate (Armor Proteines–35460)
Reddy and Mahoney, 1991a	Raw milk	
Reddy and Mahoney, 1991b	α_{s1}-casein	
Gaucheron *et al*, 1996, 1997a	Sodium caseinate	Armor Proteines F–35460
Gaucheron *et al*, 1997b	Skim milk	Obtained by warming whole milk at 40°C for 30 min
Hekmat and McMahon, 1998	Pasteurized (79°C for 28s) skim milk	

2.7.3. Binding of Iron to Casein in Milk

When iron is added to milk, it has been found that the added iron is associated largely with the milk protein, primarily caseins. Investigation by Demott and Dincer (1976) found that when iron was added to raw milk, about 85% of the added iron was bound to the casein fraction and only 9% was bound to the whey protein fraction. Studies by Carmichael *et al* (1975) showed that the casein fraction in the milk bound approximately 75% of the iron presented (as ferric NTA). Results of a study on the binding of supplemental iron to casein micelles by Hegenauer *et al* (1979c) showed similar finding with a slightly larger percentage (90%) of iron bound to the casein fraction and that the casein micelle was the principal sequestrant of iron added to milk.

It was found that saturation was reached as the free iron was added to milk. Hegenauer *et al* (1979c) claimed that at low concentrations (< 5mM Fe), nearly all iron was associated with the casein fraction; with increasing iron concentration, casein bound increasingly smaller amounts of the total iron and eventually became saturated. Carmichael *et al* (1975) showed that the iron binding by the casein reached a maximum when the caseins from 1 mL of nonfat milk bind about 2.5–2.75 μmoles Fe/mL. This iron binding process to milk proteins was relatively rapid. Carmichael *et al* (1975) reported that binding was achieved in 2 hours.

In milk, the iron binding to caseins may be different than in model solutions (e.g. sodium caseinate or individual caseins) because in milk, the phosphoserine interacts with calcium phosphate salts (Gaucheron, 2000). Most of the iron added to cow's milk binds to the phosphoserine residues in the casein micelle, while some is sequestered by inorganic phosphate in both the serum and the casein micelle (Jackson and Lee, 1992).

Addition of iron to milk caused precipitation of the proteins, especially caseins (Gaucheron *et al*, 1997b). When iron was added to skim milk (500 mL), protein precipitation occurred when iron concentration added to milk was about 8 mM (Gaucheron *et al*, 1997b).

Addition of iron ions to skim milk also resulted in pH decreased (Gaucheron *et al*, 1997b). This decrease in pH was significantly greater with added $FeCl_3$ than with $FeCl_2$. These pH decreases were related to the acidities of iron solutions and to exchanges between iron ions added and micellar bound H^+. The characterization of iron–phosphopeptide β-casein (1–25) complexes by electrospray–ionization mass spectrometry (Gaucheron *et al*, 1995) showed a release of three protons for one bound iron atom.

Distribution of iron after it was added to milk was investigated by ultracentrifugation. It was found that approximately 83–89% of added iron was bound to the colloidal phase (i.e. micellar phase) (Gaucheron *et al*, 1997b; Hekmat and McMahon, 1998). There were several possible forms of this micellar iron: iron associated with caseins and/or iron associated with colloidal calcium phosphate and/or iron associated with citrate molecules. However, these were not distinguishable by ultracentrifugation.

Upon addition of iron, the distribution of calcium, phosphate and citrate between the micellar and soluble phases also changed as the added iron increased (Gaucheron *et al*, 1997b). It was found that the calcium concentration in the soluble phase decreased slightly when skim milk was enriched with $FeCl_2$ but did not change when $FeCl_3$ was used. The inorganic phosphate concentration in the soluble phase decreased linearly and independently of the iron salt used. Thus, the micellar phase was enriched with inorganic phosphate ions and some calcium ions had probably been exchanged between the micellar phase and the soluble phase. The citrate concentration in the soluble phase decreased as a function of iron concentration and further work was recommended to find out the reasons for this behaviour (Gaucheron *et al*, 1997b).

Gaucheron *et al* (1997b) also found that the average diameter of particles was constant and unmodified in the absence or in the presence of iron. However, there was a decrease in the casein micelle hydration as a function of concentration of two different iron salts (as Fe^{2+} or Fe^{3+}).

2.7.4. Binding of Iron to Caseinate

Iron has been added to commercial caseins with sodium caseinate being the most used commercial milk proteins (Nelson and Potter, 1979; Gaucheron *et al*, 1996, 1997a). When iron is added to caseinate, there are some changes happening during the iron binding process.

Addition of iron to sodium caseinate caused precipitation of the proteins (Gaucheron *et al*, 1996, 1997a). Gaucheron *et al* (1997a) investigated the binding of Fe^{3+} ($FeCl_3$) to sodium caseinate; the authors indicated that initial charge of iron is important in the casein precipitation. When Fe^{3+} was used, the casein precipitated at iron concentration of 2 mM (as compared to 4 mM iron when Fe^{2+} was used) (Gaucheron *et al*, 1996). This precipitation would correspond to negative charge neutralization of caseins by iron or to formation of intermolecular iron bridging or a combination of the two (Gaucheron *et al*, 1996).

A decrease in pH was also observed when iron was added to sodium caseinate (Gaucheron *et al*, 1996, 1997a). The pH of sodium caseinate solution was 6.62 and there was a decrease of about 0.12 units of pH when 1.5 mM of $FeCl_2$ was added (Gaucheron *et al*, 1996). The decrease in pH was due to the acidity of iron chloride solution (pH 3.3) and to the binding of iron to phosphoseryl residues.

Binding of iron to sodium caseinate caused a release of sodium (Gaucheron *et al*, 1996). The release of sodium is due to the decrease in pH and also to the binding of iron to phosphoseryl residues. This indicated that there was possible exchange between iron and proton and between iron and sodium.

2.7.4.1. Binding sites in caseins for iron

The affinity of casein for iron is specific and is attributable to the phosphoserine residues in these phosphoproteins (Carmichael *et al*, 1975; Manson and Cannon, 1978;

Hegenauer *et al*, 1979a; Gaucheron *et al*, 1996, 1997a; Jackson and Lee, 1992; Hekmat and McMahon, 1998). Manson and Cannon (1978) showed that phosphoserine residues present in caseins rapidly catalyse the oxidation of iron from the ferrous to the ferric state with the formation of stable ferric–phosphoprotein complexes. In the iron-casein complexes, iron is probably bound to caseins via the oxygen of the phosphate group and would adopt a tetrahedral coordination structure as observed with ferric iron–saturated phosvitin (Webb *et al*, 1973).

Apart from phosphoseryl residue as the main binding site of iron, it was suspected that there are other possible binding sites. Reddy and Mahoney (1991b) found that enzymic dephosphorylation of α_{s1}-casein reduced the binding of iron but did not eliminate it. Hence, other possible binding sites that have been proposed are: carboxyl groups (glutamic and aspartic residue), phenolic group (tyrosyl residue), sulphydryl (cystedyl residue) and imidazole group (hystidyl residue) (Gaucheron *et al*, 1996, 1997b). Iron complexation to carboxyl groups in a bovine serum albumin digest was observed by Shears *et al* (1987). For the caseins (including α_{s2}-casein) both the phosphoserines and carboxyl groups can bind iron (Hekmat and McMahon, 1998).

2.7.4.2. Binding of iron to individual caseins

The binding of iron to individual proteins has been investigated. Demott and Dincer (1976) showed that added iron binds to α_{s1}-casein, β-casein and κ-casein in the ratio 72:21:4. Vaughan and Knauff (1961) showed the relative affinity of Fe^{3+} to milk proteins is: α_{s1}-casein > β-casein > BSA > κ-casein > β-Lg > α-La.

In general, those individual milk proteins that contain more phosphorylserine groups have the greatest iron-binding affinity. The reversed-phase HPLC profiles of caseinate in the presence of 1.5 mM Fe^{3+} showed that α_{s1}-casein, which have 7–9 phosphoserine residues per molecule, was more modified than β-caseins, which have 5 phosphoserine residues per molecule (Gaucheron *et al*, 1997a). It has been found that iron binds

preferentially to α_s-caseins in milk by way of an iron(III)–oxyphosphate complex on the phosphoserine residues (Hegenauer *et al*, 1979c). However, the binding of iron to α_{s2}-casein, which is the most phosphorylated of caseins (10–13 phosphoserine residues), was not observed when Gaucheron *et al* (1997b) studied the chromatography analysis of $FeCl_2$ and $FeCl_3$ added to skim milk.

Reddy and Mahoney (1991b), using ultraviolet and fluorescence spectroscopy, also showed a conformational change of bovine α_{s1}-casein in interaction with Fe^{3+} showing that the iron was bound to α_{s1}-casein. Similar modifications of α_{s1}- and β-caseins were also observed after $FeCl_2$ addition to sodium caseinate (Gaucheron *et al*, 1996). Gaucheron *et al* (1997b) concluded that, from fluorescence and chromatographic studies, a casein conformational change in the presence of iron ions and/or an association of casein molecules occur via iron bridges. These changes of protein structures seem to be independent of the initial oxidation state of iron added (Fe^{2+} or Fe^{3+}).

Baumy and Brule (1988b) studied the addition of iron ($FeCl_2$) to β-casein and showed that β-casein has a binding capacity of at least 7 cation of Fe^{2+}. Addition of Fe^{2+} to β-casein also caused precipitation and this occurs when all the sites are saturated. The maximum concentration of iron to be added before precipitation was 7 moles Fe^{2+} per β-casein mole.

2.7.4.3. Effect of pH, ionic strength, temperature and chelating agents on iron binding

Because iron-protein complexes can be used as food additives and thus undergo various treatments such as heat treatment, acidification, increase in ionic strength, it is important to test their stabilities under these conditions.

pH

The effect of changing the pH (i.e. acidification) of iron-supplemented casein solution was investigated (Baumy and Brule, 1988b; Gaucheron *et al*, 1996; Gaucheron *et al*, 1997a; Hekmat and McMahon, 1998). In general, it was found that the link between iron and caseins is stable to changes with changes in pH, although Nelson and Potter (1979) showed that the amount of iron bound to caseins changed with pH.

Baumy and Brule (1988b) found that the amount of Fe^{2+} (from $FeCl_2$) bound to β-casein was independent of pH. Between pH 5 and 8, it was found that the number of bound Fe^{2+} per mole of protein was the same and the binding ratios (amount of mineral bound to protein/total amount of mineral) was also the same. Reddy and Mahoney (1991a) also found that the number of iron binding sites on α_{s1}-casein was independent of pH over the range pH 5.5–6.6.

Gaucheron *et al* (1996) studied the acidification of Fe^{2+}-casein complexes in the pH range 6.5 to 3.7 and found that the bound iron concentration was not affected. Furthermore, the behavior of Fe^{3+}-casein complex under acidification (pH range from 6.8–2.5) was also similar to iron(II)–casein complex. There was no release of Fe^{3+} observed (Gaucheron *et al*, 1997a).

The stability of iron-casein complexes at different pH indicated that the iron is probably bound to caseins by coordination bonds (with NH_2, CO_2H, CONH, and H_2O) and not by ionic bonds (Gaucheron *et al*, 1996). This suggested that iron would not be released from phosphoseryl residues of caseins by chemical or biological acidification of iron-supplement milk or iron-supplemented dairy products.

The effect of changing the pH in skim milk-fortified with iron was studied by Gaucheron *et al* (1997b) and Hekmat and McMahon (1998). When the pH in milk was lowered, colloidal calcium phosphate (which is associated with casein micelles at the normal pH of milk) was solubilised. Hence, there was a change in the micelle structure resulting from the loss of calcium and phosphate from the micelles. In the presence of

iron, both authors found that the amount of calcium and phosphate which solubilised from the casein micelle was the same as the one when the milk had no added iron.

Hekmat and McMahon (1998) found that the presence of iron did not affect the overall behaviour of calcium and phosphate (under acidification) except to delay the release of calcium and phosphate from the casein micelles. This was due to the significantly higher calcium and phosphate contents in the serum at pH 6.2, 5.8, and 5.3 in the skim milk without iron added. The probable reasons of why iron-fortification caused the colloidal calcium phosphate of milk to be slightly more resistant to solubilisation as the pH was lowered were the formation of iron coordination complexes with colloidal calcium phosphate which allowed more phosphate to be retained through ionic bonding (at pH 6.2, 5.8, and 5.3). The other reason could be that the iron provided the complexing sites for calcium that are less pH dependent. They also claimed that the iron binding to milk proteins was independent of pH in the range 4 to 6.7.

On the other hand, Gaucheron *et al* (1997b) found that the iron was never solubilised in the pH range of 6.7 to 4.0. Hence, they claimed that there was no association of iron ions to colloidal calcium phosphate because iron ions were not solubilised at the same time as calcium and inorganic phosphate ions. The absence of iron solubilisation indicated that this element would not be released of caseins by chemical or biological acidification of iron–supplemental milk or iron-supplemented dairy products.

Ionic strength

Ionic strength is very important for the stability of colloidal particles and for the conformation of proteins (Walstra and Jenness, 1984). Hence, change in ionic strength of the iron-casein complex could affect the binding of iron to the caseins. However, it was found that the change in ionic strength did not affect the binding of iron to caseins (Baumy and Brule, 1988b; Gaucheron *et al*, 1997a).

Study by Baumy and Brule (1988b) found that iron (as $FeCl_2$) bound to β-casein was not dependent on the ionic strength. Gaucheron *et al* (1997a) also found that Fe^{3+}

bound to caseins was not affected by changes in ionic strength (0–0.12 M) of the solution. This showed that casein molecules bound the added iron strongly, probably through the coordination links of the iron and the protein (Baumy and Brule, 1988b; Gaucheron *et al*, 1997a).

Temperature

Studies by Gaucheron *et al* (1996 and 1997a) on the stability of iron-casein complexes from sodium caseinate using both $FeCl_2$ and $FeCl_3$, showed that these complexes (with 1.5 mmol iron/L) were not affected by heat treatments (50, 70 and 90°C for 15 min). The same result was also obtained when iron-supplemented skim milk was investigated by Gaucheron *et al* (1997b), regardless of the initial state of iron source used ($FeCl_2$ or $FeCl_3$) (at 90°C, 15 min).

Chelating agents

When the iron-casein complex was incubated in the presence of metal–chelating agent, such as EDTA disodium salt and trisodium citrate, there was a significant release of iron (Gaucheron *et al*, 1996). This showed that there was a reversibility of the link between casein and iron. It may be noted that EDTA disodium salt added to food enhances iron bioavailability by chelating added iron and that it could be used in milk or dairy products (Gaucheron *et al*, 1996).

2.7.5. Iron Binding to Whey Proteins

Iron has also been shown to complex with whey proteins although in general, whey protein fraction has a lesser binding affinity than the casein fraction.

Studies by Jones *et al* (1972 and 1975) showed that a liquid complex ferric ion with a long chain phosphate could be made from ferric chloride and sodium polyphosphate. This ferripolyphosphate complex could precipitate virtually all the protein in commercial acid whey at pH 3.2–4. This ferripolyphosphate–whey protein powder was then lyophilised and was claimed as an effective iron supplement in flour, milk, and

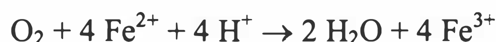
cereal products. Another iron-whey protein complex that has been made was ferrilactin, which was a complex formed between ferric iron and whey proteins under acidic conditions, as reported by Jackson and Lee (1992). Amantea *et al* (1974) also found that a soluble ferric whey protein containing greater than 80% protein with excellent amino acid composition and functional properties could be prepared by an improved heating method at acid pH.

Individual whey proteins such as α-La and β-Lg have been reported to bind iron (Fe^{2+}) (at pH 6.6, ionic strength < 0.01 M) (Baumy and Brule, 1988a). The binding ability of α-La was 6 ions of Fe^{2+} while β-Lg had binding ability of > 3.5 ions of Fe^{2+}. The affinity was lower when the pH was decreased from 6.6 to 5.0 or when the ionic strength increases to 0.1 M.

2.7.6. Problems with Oxidation Due to Addition of Iron

Bioavailable forms of iron are chemically reactive and often produce undesirable effects when added into the diet. In iron-fortified foods, the ferrous form of iron will go into its ferric state. This change can cause oxidative deterioration, which results in undesirable odors and flavors. The thiobarbituric acid (TBA) test has been extensively applied to raw milk in which the absorbance of TBA reaction products correlates positively with organoleptic evaluation (Gaucheron, 2000).

The formation of iron–casein complexes induces the oxidation of iron from the ferrous to the ferric state (Manson and Cannon, 1978) according to the reaction:



$$O_2 + 4\,Fe^{2+} + 4\,H^+ \rightarrow 2\,H_2O + 4\,Fe^{3+}$$

The characteristics of different iron complexes as donors to caseins were investigated by Hegenauer *et al* (1979a). It was found that lipid peroxidation in iron–supplemented milk was significantly lower when the milk was supplemented rationally with chelated forms of iron, such as ferric EDTA, ferric NTA, ferric fructose, ferric lactobionate and ferric polyphosphate, as compared to ferrous sulphate. The iron exchange between ferric–NTA chelate with casein phosphoserine residue meant that iron was bound more

strongly than the original complex. This chelation served to remove iron from the environment of the oxidisable milkfat. Hence, they had the least tendency to oxidise milkfat. Such iron complexes may thus be more suitable iron donors for fortification in order to preserve the organoleptic stability of milk (Carmichael *et al*, 1975; Hegenauer *et al*, 1979a).

Hegenauer *et al* (1979a) also found that in milk, the catalytic effect of iron on oxidation was greater if the iron was in the ferrous state rather than in ferric state. This was because binding of the casein fraction of raw milk to Fe^{2+} to form Fe(III)–oxyphosphate chelates after autoxidation was incomplete. The affinity of Fe^{2+} for the milk fat will then contribute to the rapid peroxidation. This tendency for Fe^{2+} to undergo cyclic autoxidation and to form ferric hydroxide-protein complexes may contribute additional instability to supplemented milks during prolonged storage (Hegenauer *et al*, 1979a).

Hekmat and McMahon (1998) found that oxidation of the fat occurred in milk when it was fortified with ferrous sulfate and ferrous ammonium sulfate. This oxidation was reduced by using a chelated form of iron for milk fortification. They suggested that if the iron was bound and unable to move between the ferrous and ferric states (so that it cannot catalyse oxidation), the oxidative deterioration could be prevented.

2.7.7. Application of Iron–Protein Complexes

A few studies have investigated the application of the iron-protein complexes by adding them into food products such as cheddar cheese (Zhang and Mahoney, 1989a and 1989b), Mozarella cheese (Rice and McMahon, 1998) and chocolate milk (Douglas *et al*, 1981).

Zhang and Mahoney (1989a and 1989b) used $FeCl_3$, ferric citrate, iron-casein complex, and ferrypolyphospate-whey protein complex to fortify cheddar cheese. It was found that iron recoveries in the cheeses were 71–81% for $FeCl_3$, 52–53% for ferric citrate, 55–75% for iron-casein complex, and 70–75% for ferripolyphosphate–whey protein

complex and that these iron sources (except for ferric citrate) were potential iron fortification sources for cheese. Zhang and Mahoney (1989b) found that the bioavailability of cheese fortified with iron-casein complex, ferripolyphosphate–whey protein complex and iron–whey protein complex was similar to $FeSO_4$.

$FeCl_3$, iron–casein complex and iron–whey protein complex were used by Rice and McMahon (1998) to fortify Mozarella cheese at a level of 25 or 50 mg Fe/kg of cheese. It was found that fortification of milk with $FeCl_3$ prior to renneting was simple, and the use of a protein–chelated iron source did not provide any advantages with regard to fat oxidation that would justify the additional expense and time required for their preparation. Furthermore, more iron was retained in the cheese when $FeCl_3$ was used. There was no increase in chemical oxidation between the control and iron-fortified cheeses. Slight increases in metallic and oxidized flavors were observed in the iron-fortified cheese.

Douglas *et al* (1981) studied the fortification of eleven different iron compounds to chocolate milk. It was found that all other added compounds resulted in initial and persistent off-colors. However, sodium ferric pyrophosphate, ferripolyphosphate, and ferripolyphosphate–whey protein complex produced little or no off-color change in the products initially and after 2 weeks of storage. Flavor evaluation showed that ferric compounds produced little or no off-flavors in chocolate milks initially or after holding at 4°C for 7 and 14 days. However, ferrous compounds produced off-flavors initially, but flavor scores improved after milks were held at 4°C for 14 days.

Yoghurts (non-fat and low fat) were fortified with 10, 20, and 40 mg Fe/kg of yoghurt (Hekmat and McMahon, 1997). It was claimed that there was no increase in detection of chemical oxidation and that score for 'oxidised, metallic, bitter and other off-flavours' by trained panelists were 'not very perceptible'. The consumer panel did not detect any differences in the appearance, mouthfeel, flavour or overall quality between fortified and unfortified flavoured yoghurts.

More possible applications of iron-protein complex were reviewed by Gaucheron (2000). Products that were mentioned were brown whey cheese, white soft cheese, Baker's and cottage cheese, Harvati cheese and chocolate drink powders.

2.8. Zinc Binding Studies

2.8.1. Zinc in Bovine and Human Milk

Large variations in the mean zinc concentration in cow's and human milk have been reported (Flynn and Cashman, 1997). Zinc content of human milk is in the range of 0.65–5.3 mg/L whereas in cow's milk the concentration is in the range of 2–6 mg/L.

In cow's milk, most of the zinc is in the skim milk fraction, with only 1–3 % in the lipid fraction. From ultracentrifugation, it was found that 95% of the zinc in the skim milk fraction was associated with the casein micelles (Blakeborough *et al*, 1983; Singh *et al*, 1989b). When analysed on column Sephadex G-150, Blakeborough *et al* (1983) found that the micelles were of very high molecular weight and predominantly consisted of α-casein–κ-casein and α-casein–β-casein complexes.

In human milk, 29% of the zinc is associated with fat fraction, 14% to the casein micelles, 28% to whey proteins and 29% to a low molecular weight fraction (Singh *et al*, 1989a). Further analysis by Blakeborough *et al* (1983) showed that zinc in human milk was associated with protein of high molecular weight which was identified as lactoferrin. Lactoferrin is a major glycoprotein component in human milk, of subunit MW 75,000 Da and is structurally distinct from caseins. It acts as an iron-binding protein and has been reported to be a bacteriostatic agent. However, there was no clear characterization of lactoferrin as the primary zinc-binding protein of human milk because there was little zinc associated with the protein as purified by affinity chromatography on heparin–Sepharose 6B.

Low molecular weight zinc-binding ligand

Investigations have found that a small proportion of the zinc in both cow's and human milk was associated with low molecular weight compounds (Cousins and Smith, 1980; Blakeborough *et al,* 1983; Martin *et al*, 1981, 1984; Singh *et al*, 1989b). Singh *et al* (1989b) claimed that approximately 5% of zinc was associated to these fractions and Blakeborough *et al* (1983) showed that these fractions bound 10% of the zinc in human milk.

Cousins and Smith (1980) (using chromatography analysis on column Sephadex G-75) showed that association of zinc with low molecular weight protein fractions in fat-free cow's and human milk was elevated when extra zinc (189 μg Zn^{2+}) was added *in vitro*. They found that the distribution of zinc might be influenced by the total amount of protein present in cow's and human milk as well as the type of protein. In human milk, added Zn^{2+} was found to be more associated with both high and low molecular weight fraction of the milk, especially the low molecular fraction. In cow's milk, added Zn^{2+} was found to be eluted with the low molecular weight fraction as compared to the absence of non-protein zinc complex of low molecular weight fraction from non-incubated cow's milk. Similar findings were reported by Blakeborough *et al* (1983). The association of zinc with the protein fraction in the milk was also affected by the content of the zinc in the milk sample. The observation that proteins from cow's milk bind more zinc before a zinc binding ligand (ZBL) is detected, compared to human milk, supports that contention (Cousins and Smith, 1980).

It was postulated that zinc was only bound to low molecular weight fractions after the binding sites of higher molecular weight ligands become saturated with zinc (Cousins and Smith, 1980). In addition, the fact that zinc did not preferentially bind to the low molecular weight ligands, i.e. ZBL, compared to high molecular weight sites, suggested that this zinc was loosely bound. Zinc bound in this fashion would be expected to exhibit higher bioavailability than zinc associated with certain proteins. Therefore, it was understandable that ZBL–bound zinc was more highly available (Cousins and Smith, 1980).

A considerable research has been directed at identifying the low molecular weight metal binding ligands in milk. Two different organic acids capable of binding zinc were reported to be present in ultrafiltered human milk. They are citric acid (which is present in both human and bovine milk) and picolinic acid (a metabolite of tryptophan) (Martin *et al*, 1981).

The study of metal binding ligands of human and cow's milk sample to copper and zinc using the modified gel chromatography technique showed that there was only one metal binding peak which matched the zinc(II)citrate system. No Zn^{2+} was associated with picolinic acid (Martin *et al*, 1981). Martin *et al* (1984) has identified this component to be citrate using modified gel chromatography and NMR spectrophotometry method.

2.8.2. Binding of Zinc to Casein in Milk

In vitro addition of zinc to milk showed that zinc bound mostly to casein fraction and only a minor percentage of the added zinc was bound by the whey proteins (Harzer and Kauer, 1982; Blakeborough *et al*, 1983; Singh *et al*, 1989a, 1989b).

When zinc binding capacity of caseins was investigated by Harzer and Kauer (1982) using equilibrium dialysis (pH 7.4) (final protein concentration of 5 mg protein/mL), it was found that caseins were able to bind the added zinc with a binding capacity of 8.4μg zinc/mg casein. It was in excess of zinc content in cow's milk (2–5 μg/ml). Singh *et al* (1989a) found that bovine casein had zinc-binding capacity of about 5.6 atoms of Zn/mol protein, which is considerably higher than that reported by Harzer and Kauer (1982) (~ 3 atoms Zn/mol protein). Singh *et al* (1989a) also found that human casein had a binding capacity of 8 atoms Zn/mol protein.

Increased addition of zinc to caseins caused precipitation of the protein. When zinc was added to bovine casein at a concentration of > 150 μg Zn/mL, precipitation of protein and zinc occurred upon dialysis (Harzer and Kauer, 1982).

The nature of zinc binding to bovine casein micelles was investigated by Singh *et al* (1989b). They found that there were two distinct micellar Zn fractions. 32% of Zn in skim milk was directly bound to caseins and ~ 63% was associated with the colloidal calcium phosphate (CCP). Zinc directly bound to caseins was readily removed by EDTA and this casein-bound fraction corresponds to the loosely bound Zn fraction in skim milk. The remainder appears to be more tightly bound to CCP and was solubilised only when the casein micelles disintegrate.

2.8.3. Binding of Zinc to Caseinate

Addition of zinc to commercial sodium caseinate was studied by Gaucheron *et al* (1997a). It was found that when zinc was added to caseinate, the casein fraction was able to bind the added zinc and the binding caused precipitation of the casein. Gaucheron *et al* (1997a) explained that this precipitation corresponds to negative charge neutralisation of casein molecules by the cations added with a decrease in the electrostatic repulsions between the proteins and thereby an increase in interactions between the hydrophobic regions of proteins.

A decrease in pH was found when zinc was added to sodium caseinate (Gaucheron *et al*, 1997a). This was due to the acidity of the $ZnCl_2$ solutions and the release of protons consequently to exchanges multivalent cations-protons during the binding of cations to casein molecules.

2.8.4. Binding of Zinc to Individual Caseins

Singh *et al* (1989a) studied the binding of zinc to purified bovine caseins. They found that among the individual casein proteins, bovine α_{s1}-casein had the greatest zinc binding capacity followed by β- and κ-caseins. Table 2.4 shows the number of binding sites and their average association constants. A close similarity in the nature of zinc binding to the different individual caseins was indicated by the finding that all log K_{app}

values (the apparent average association constant) were in the range 3–3.2 (Singh *et al*, 1989a).

Precipitation was reported to occur when zinc was added to α_{s1}-casein (Singh *et al*, 1989a). Protein aggregation was observed as zinc concentration was increased and the protein precipitated at a free zinc concentration of 1.7 mM. The observed aggregation of caseins with increasing zinc concentration supported the explanation of the Scatchards plot for zinc binding to caseins (Singh *et al*, 1989a), which showed upward convexity at low molar ratios of binding for all caseins.

Possible competition between calcium and zinc for binding to α_{s1}-casein was investigated by equilibrium dialysis (Singh *et al*, 1989a). Displacement of bound-Ca by zinc (but not the reverse) was observed, indicated that zinc and calcium compete for the same binding sites and that zinc was bound much more strongly than calcium.

Investigation of zinc binding to purified β-casein (Baumy and Brule, 1988b) found that β-casein bound the added zinc and there were two different sites of zinc binding; the first 5 sites had higher affinity than the other 2 following sites. Addition of zinc to β-casein also caused precipitation of the protein and Baumy and Brule (1988b) found that the maximum concentration of zinc which did not give precipitation was about 7 moles (4.9×10^{-4} mol/L) of Zn^{2+} per β-casein mole. They postulated that precipitation occurs when all the binding sites in the proteins were saturated.

Table 2.4. Apparent average association constant K_{app} and numbers of binding sites for Zn binding to bovine proteins (Singh *et al*, 1989a).

Bovine Proteins	Log K_{app}	Number of sites
α_{s1}-casein	3.00	11
β-casein	3.15	8
κ-casein	3.00	2

2.8.5. Binding Sites

In the zinc–casein complex, zinc was bound to the negatively charged phosphate group in the casein fraction (Harzer and Kauer, 1982; Singh *et al*, 1989a; Baumy and Brule, 1988b; Gaucheron *et al*, 1997a). This was supported by the findings of Harzer and Kauer (1982) that at pH 2, there was no zinc–casein binding because in acid, the phosphate group was in charge-free state. Also, when zinc was added to dephosphorylated casein, there was no zinc–casein binding. Singh *et al* (1989a) found that dephosphorylation of bovine α_{s1}-casein reduced zinc binding capacities. However, the number of phosphoserine residues in α_{s1}-casein was only 8 and the total number of binding sites for zinc on α_{s1}-casein was 11 (Table 2.4). This indicated that there were other possible binding sites for zinc, such as carboxylic group or histidine residues. Baumy and Brule (1988b) stated that Scatchards plot concerning the binding of Zn to β-Casein at pH 6.6 and ionic strength of 0.1 showed that phosphoserines have a 4.5 times higher affinity than carboxylic sites.

2.8.6. Effect of pH, Ionic Strength and Chelating Agents on Zinc Binding

Changes in pH, ionic strength and different chelating agents in zinc-casein complexes have been investigated to find out the stability of these complexes.

Effect of pH

There was a release of zinc ions from zinc-casein complexes during acidification i.e. decrease in pH in the range 5–8 (Baumy and Brule, 1988b) and 6.8–2.5 (Gaucheron *et al*, 1997). Harzer and Kauer (1982) reported that acidifying the casein–zinc complex to pH 4.6 caused complete protein precipitation and a total release of zinc into the supernatant. Singh *et al* (1989a) expressed it in terms of increase in zinc binding with increased in pH in the range of 5.4–7.0.

The decrease of zinc binding by casein molecules during acidification was due to a decrease in ionization state of the phosphoserine and histidine residues (Singh *et al*, 1989a; Gaucheron *et al*, 1997a) and indicated that the nature of link between casein molecules and zinc were electrostatic (ionic links) (Baumy and Brule, 1988b; Gaucheron *et al*, 1997a). Harzer and Kauer (1982) also claimed that zinc was only released by acid precipitation and not as a result of casein curd formation with rennet or by Ca^{2+} precipitation.

Similar phenomenon also occurred when skim milk with added zinc was acidified (Singh *et al*, 1989b). In this case, acidifcation of milk (to pH 4.6) progressively dissolved the CCP and hence reduced the binding of divalent cations to casein. These changes were reversible when the pH was adjusted back to pH 6.7, i.e. CCP was reformed on re-neutralisation of acidified milk.

Effect of ionic strength

Zinc binding was found to decrease with increasing ionic strength in the range of 0–0.1 M NaCl (Singh *et al*, 1989a) and this small decrease in binding was probably due to the changes in the activity of zinc. The same result was found by Baumy and Brule (1988b) who reported that the number of binding sites of β-casein for zinc at pH 7 and $\mu = 0.1$ was more reduced than the maximal number at pH 7 and $\mu = 0$. They reported that increase in ionic strength reduces the apparent pK of the second ionization of phosphoserine residues; thus they were more dissociated but had a lower affinity towards zinc.

On the contrary, Gaucheron *et al* (1997a) claimed that increasing NaCl concentration in the range 0 to 0.12 M of the caseins containing 1.5 mM-cations did not affect the amount of zinc bound to caseins. This was because casein molecules have better affinities for zinc and bound zinc strongly, hence exchanges between sodium ion and zinc ions did not occur.

Effect of different chelating agents

When zinc-casein complex was dialyzed against buffer and metal chelating agents (EDTA, citrate, picolinic acid, tartrate), most of the zinc was released from the protein. However, when the complex was dialyzed against buffer only (Tris–HCl buffer), there was no zinc release. This indicated that zinc-casein interaction was rather strong (Harzer and Kauer, 1982).

When zinc–casein complex was digested (tryptic digestion), then separated on Sephadex G-75, it was found that the zinc remained bound to phosphopeptides. However, when citrate was present, zinc was no longer present together with these peptides but found to be a component with low molecular weight (i.e. citrate coeluted with zinc, as zinc–citrate complex) (Harzer and Kauer, 1982).

Addition of EDTA to zinc bound in casein micelles did not cause a release of zinc which bound in the CCP fraction. However, for the zinc bound in the casein, addition of 0.2 mM EDTA (to CCP-free milk) caused a decrease in binding (i.e. zinc was rendered soluble) showing that the casein-bound zinc is loosely bound zinc fraction in casein micelles (Singh *et al*, 1989b).

2.8.7. Binding of Zinc to Whey Proteins

There are very few detailed studies done on the zinc binding to whey proteins. Most of the literature only stated the general contention that whey proteins have lower zinc-binding ability than caseins (Cousins and Smith, 1980; Harzer and Kauer, 1982; Blakeborough *et al*, 1983; Singh *et al*, 1989a).

Studies of zinc binding to individual whey proteins from bovine milk by Singh *et al* (1989a) showed that the major whey proteins, i.e. β-Lg and α-La, showed little capacity for zinc binding compared with caseins. However, BSA bound significant amounts of zinc (> 8 atoms Zn/mol protein). Lactoferrin also bound a maximum of 0.4–0.6 atoms of Zn/mol protein. They reported that BSA, β-lg, and α-la showed similar zinc-binding

behaviour to that of dephosphorylated caseins, with at least two distinct classes of binding sites.

According to Baumy and Brule (1988a), the affinity of α-La and β-Lg for zinc is high, with binding ability of 4.5 ions of zinc for α-La and 2.5 ions of zinc for β-Lg. The affinity becomes lower when pH decreases (from 6.6 to 5.0) or when the ionic strength increases (from < 0.01 M to 0.1 M).

A study by Ren *et al* (1993) on the binding of zinc to human α-La using high resolution x-ray crystal structure showed that zinc ion can bind at a specific site in α-La with no significant secondary structural changes. The coordination of zinc ion involved a symmetry-related molecule in the crystal, the crystal contact being stabilized by a sulfur (SO_4^{2-}) ion bound at the interface between three molecules. Also, α-La has several relatively strong zinc binding sites, which were filled sequentially, the process being accompanied by protein aggregation.

2.9. Oxidation

As explained in Section 2.7.6, addition of minerals to food can cause oxidative deterioration, especially when iron was used. It has been found that iron–complex was` more suitable as iron donors for fortification in order to prevent oxidative deterioration. Hence, it is deemed important to review the process of oxidation, lipid oxidation catalysed by the presence of metal ions and some of the methodologies used to investigate the extent of oxidation.

Lipid peroxidation is a major cause of food deterioration, leading to loss of functional properties, such as development of various off flavours and off odours generally called rancid (oxidative rancidity). In addition, oxidative reactions can decrease the nutritional value of food and certain oxidation products are potentially toxic (Nawar, 1996; Yen *et al*, 1999).

From the standpoint of food oxidation, the important lipids are the unsaturated fatty acids moieties, particularly oleate, linoleate, and linolenate. Simpler model systems using these fatty acid moieties have been used to ascertain mechanistic pathways because of the complexity in oxidative reactions in food lipids (Nawar, 1996). The susceptibility and rate of oxidation of these fatty acids increase in a somewhat geometric fashion in relation to their degree of unsaturation (Gray, 1978).

The main reaction involved in oxidative deterioration of lipids is generally agreed as 'autoxidation' where the pathway involves a self-catalytic free radical chain reaction with molecular oxygen (Nawar, 1996). In the presence of oxygen, the unsaturated fatty acids in fats and oils undergo oxidation and form odorless and tasteless hydroperoxides (ROOH) through the free radical mechanism. The overall mechanisms consist of three phases: (1) *initiation*, the formation of free radicals; (2) *propagation*, the free-radical chain reactions; and (3) *termination*, the formation of non-radical products.

Because production of the first few radicals (initiation) necessary to start the propagation reaction is thermodynamically difficult, this reaction must be catalysed. It has been proposed that initiation of oxidation may take place by hydroperoxide decomposition, by metal catalysis (often transition metal), or by exposure to light and heat (Ward, 1985; Nawar, 1996, Yen *et al*, 1999).

Hydroperoxides, the primary initial products of lipid autoxidation, have profound significance in this process because they may react with other food constituents such as protein. They are unstable and decompose to yield carbonyl compounds such as short chain aldehydes, ketones and other oxygenated compounds which are considered to be responsible for the development of rancidity in stored foods. These carbonyl compounds often have unpleasant flavours that can be detected at very low concentration (1μg per g fat) (Botsoglou *et al*, 1994; Joshi and Thakar, 1994; Yen *et al*, 1999). The overall mechanism of the lipid oxidation process is shown in Figure 2.6.

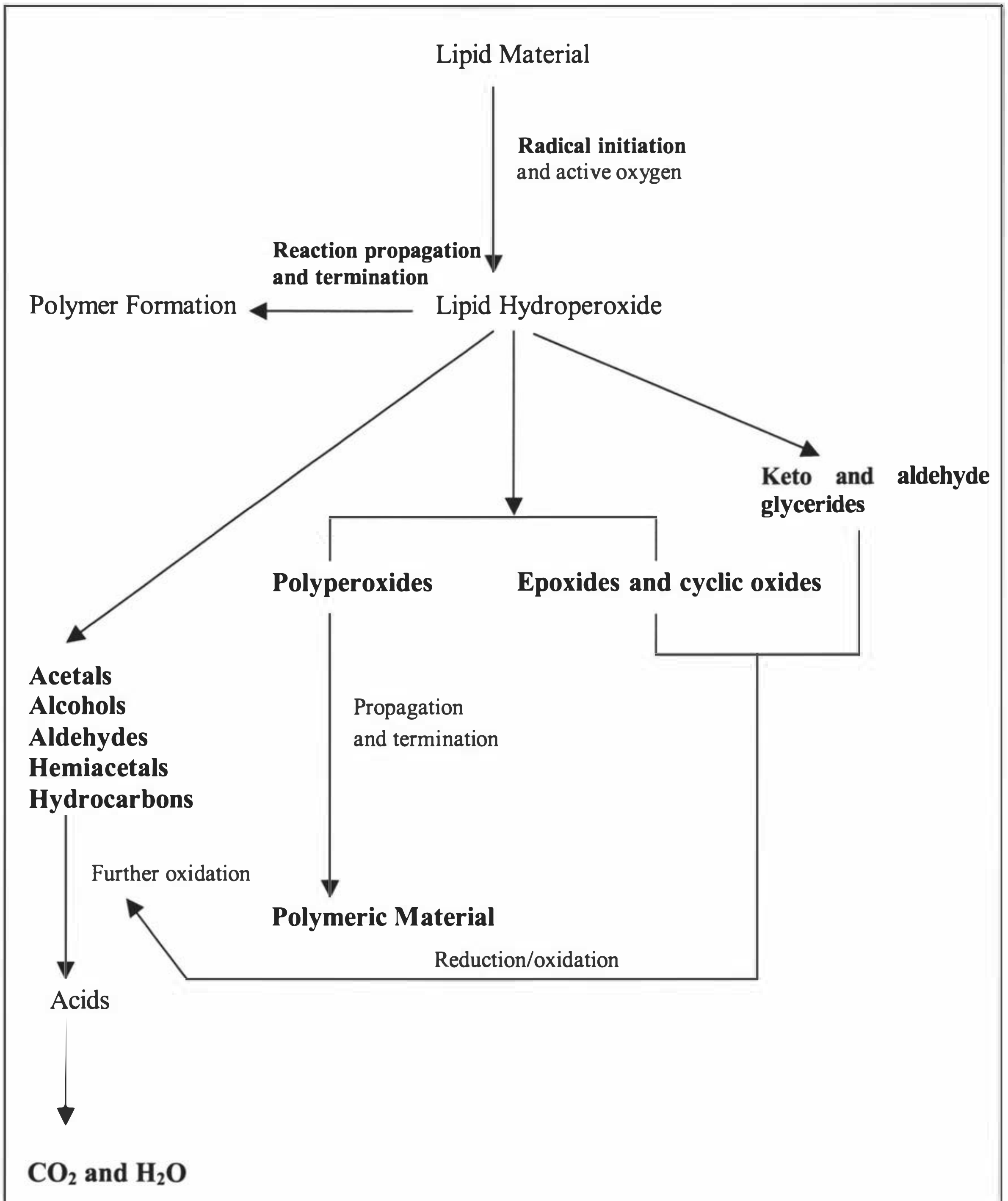


Figure 2.6. Overall mechanisms of lipid oxidation process (from Ward, 1985)

Among the several factors that could influence the rate of oxidation of lipid is the presence of metal ions in the food system. Metal ions, particularly transition metals, which possess two or more valency states and a suitable oxidation-reduction potential between them (e.g., cobalt, copper, iron, manganese, and nickel) are effective pro-oxidants (Labuza, 1971). They function primarily by decomposing hydroperoxides to generate new reaction chains (O'Connor and O'Brien, 1995). If present, even at concentration as low as 0.1 ppm, they can decrease the induction period and increase the rate of oxidation. Several mechanisms for metal catalysis of oxidation have been postulated (Nawar, 1996):

1. Acceleration of hydroperoxide decomposition:

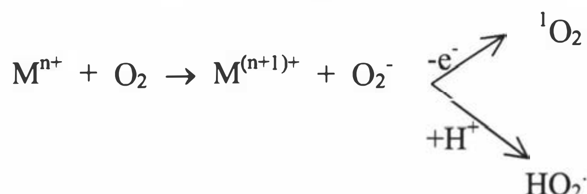
$$M^{n+} + ROOH \rightarrow M^{(n+1)+} + OH^{-} + RO^{\cdot}$$

$$M^{n+} + ROOH \rightarrow M^{(n-1)+} + H^{+} + ROO^{\cdot}$$

2. Direct reaction with the unoxidised substrate:

$$M^{n+} + RH \rightarrow M^{(n-1)+} + H^{+} + R^{\cdot}$$

3. Activation of molecular oxygen to give singlet oxygen and peroxy radical:



$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{-} \quad \xrightarrow{-e^{-}} {}^{1}O_2 \quad \xrightarrow{+H^{+}} HO_2^{\cdot}$$

2.9.1. Measurement of Lipid Oxidation

Many chemical and physical methods have been developed to quantify oxidative deterioration. Routine procedures to assay the extent of oxidation in lipids and lipid-containing foods should be simple, reliable and sensitive. However, because of the complexity of lipid oxidation, no single test can be equally useful at all stages of the oxidative process (O'Connor and O'Brien, 1995). The tests can range from simple organoleptic evaluations to chemical and physical methods. Some of the chemical methods available include peroxide value test, TBA test, total and volatile carbonyl

compounds test and oxirane determination. The physical methods include conjugated diene methods, fluorescence method, infrared spectroscopy, polarography, gas chromatography and refractometry. These procedures have been reviewed (Gray, 1978; Frankel, 1993 and Joshi and Thakar, 1994).

2.9.1.1. Sensory evaluation

One of the methods, which could provide useful information to relate to consumer acceptance of the food product, is the sensory methods based on odour and flavour evaluations (Frankel, 1993). Although these methods are very sensitive, they are time consuming and highly dependent on the quality of training the taste panel received.

2.9.1.2. Peroxide value

Measurement of hydroperoxides is the classical method for quantifying lipid oxidation and a variety of assay procedures exist. The oxidation of ferrous to ferric iron by hydroperoxides in the presence of ammonium thiocyanate to produce ferric thiocyanate, which can be quantified spectrophotometrically at 505 nm, has been used extensively to study lipid oxidation in milk. A second assay procedure is based on the reaction of oxidized fat with 1,5-diphenyl-carbohydrazide to yield a red–coloured product. A third procedure is based upon the liberation of iodine from potassium iodide by hydroperoxides (O'Connor and O'Brien, 1995).

2.9.1.3. TBA method

Thiobarbituric acid (TBA) method is based on the colour reaction between TBA and oxidation products of polyunsaturated lipids (Frankel, 1993). The reaction produces a red (532 nm absorbing) pigment by heating a mixture of TBA and sample in a strongly or mildly acidic medium. In this reaction, the red chromagen is formed through the condensation of two molecules of TBA with one molecule of malonaldehyde, which is a secondary product in the oxidation of polyunsaturated fatty acids (Gray, 1978). However, malonaldehyde is not always present in all oxidized systems (Gray, 1978; Nawar, 1996) and that aldehydes other than malonaldehyde contribute to the TBA-reactivity (Kosugi *et al*, 1991).

Many alkanals, alkenals, and 2,4-dienals produce a yellow pigment (at 450 nm) in conjunction with TBA, but only dienals produce a red pigment (at 530 nm). It has been suggested that measurement at both absorption maxima is desirable (Nawar, 1996).

TBA test is reported to be more sensitive when used with polyunsaturated fatty acids containing three or more double bonds (Gray, 1978; Frankel, 1993; Nawar, 1996). The mechanism of the TBA reaction is shown in Figure 2.7. It has been suggested that radicals with a double bond β to the carbon bearing the peroxy groups (which can only arise from acids containing more than 2 double bonds) cyclize to form peroxides with five-membered rings. These then decompose to give malonaldehyde (Gray, 1978; Nawar, 1996).

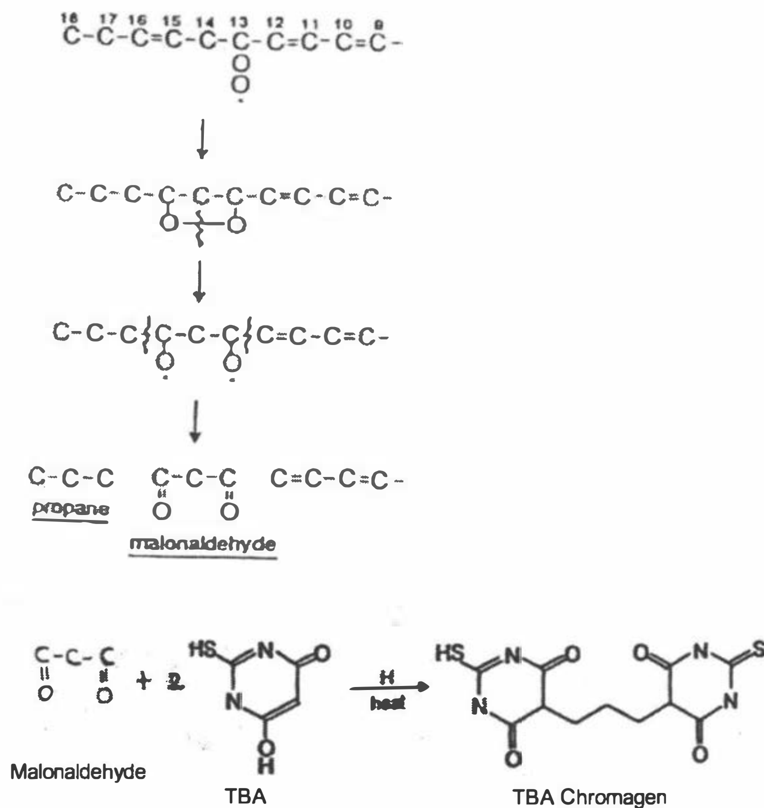


Figure 2.7. Proposed TBA reaction mechanism (from Nawar, 1996).

However, Tamura and Shibamoto (1991) analysed the lipid peroxidation products and reactive aldehydes formed from arachidonic acid, linoleic acid, linolenic acid, and oleic acid upon Fe^{2+}/H_2O_2 oxidation. They found that malonaldehyde formed upon oxidation of Fe^{2+}/H_2O_2 from linoleic acid (and also from their ethyl esters). It was also previously reported that 1 g of non-treated linoleic acid contained 14 μg of malonaldehyde. The addition of an oxidizing agent increased malonaldehyde and 4-hydroxy nonenal formation significantly.

The TBA test can be performed in several ways, however the procedure used in one experiment can be modified depending on the nature of the sample. Gray (1978) and Botsoglou *et al* (1994) mentioned some ways of performing TBA test. Because the amount of red color developed and extracted is influenced by a number of factors involved in the test (e.g. the kind of acid used for acidification, pH, heating time), standardization of a suitable procedure is necessary in performing TBA test.

It has been reported that some substances could interfere with the formation of the TBA color complex. Browning reaction products, protein and sugar degradation products were some substances, which have been reported to overestimate the extent of oxidation (Frankel, 1993). Sucrose and some compounds in woodsmoke react with TBA to give red color formation; a mixture of acetaldehyde and sucrose when subjected to the TBA test produced a 532 nm absorbing pigment identical to that produce by malonaldehyde and TBA (Gray, 1978; Nawar, 1996). When TBA test was applied to milk, lactose was found to contribute considerable interference in the TBA reaction (King, 1962). Abnormally low TBA values can also result if some of the malonaldehyde reacts with proteins in an oxidizing system (Nawar, 1996).

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

The minerals used in the binding experiments were ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) and zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), obtained from BDH Chemicals, Poole, England. HEPES Buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and certified iron and zinc standard solutions (Spectrosol) were obtained from BDH chemicals, Poole, England. The chemicals used for preparation of electrophoresis buffers were obtained from Bio-Rad Laboratories, Hercules, CA 94547, USA.

Linoleic Acid ($C_{18}H_{32}O_2$) in sealed vials was purchased from Sigma Chemical, USA. The vials were stored at -18°C. Other chemicals used for the fat oxidation test were purchased from BDH Chemicals and Univar, Ajax Chemicals, Sydney, Australia. All chemicals used were of analytical grade.

Water was purified using a MilliQ-system (Millipore Corp., Bedford, MA01730, USA).

3.1.2. Protein Powders

Sodium caseinate (Alanate 180), milk protein concentrate (MPC 85) and whey protein isolate (WPI 95) were obtained from Fonterra, Palmerston North, New Zealand. The composition of these powders is shown in Table 3.1.

Table 3.1. Composition of sodium caseinate, milk protein concentrate and whey protein isolate powders.*

Chemical	Concentrations (g/100 g powder)		
	Sodium Caseinate	MPC	WPI
Protein	93.1	84.5	93.9
Fat	0.6	1.5	0.3
Moisture	4.8	4.8	4.6
Ash	~1.5	7.4	1.9
Lactose	0.5	3.0	< 0.5

* Data from Fonterra, Palmerston North

Protein powders were dissolved in HEPES buffer at a concentration of 2%. The concentrations of the minerals in the protein solutions were determined to ensure that the iron and zinc contents were low in these solutions.

Table 3.2. Mineral compositions of sodium caseinate, milk protein concentrate and whey protein isolate dissolved in 50 mM HEPES buffer at a 2% concentration.# The mineral concentrations are expressed as mg/g powder.

Mineral	Concentration (mg/g powder)*		
	Sodium Caseinate	MPC	WPI
Ca	0.3	22.05	0.835
Cu	0.0025	0.002	0.001
Fe	0.005	0.0065	0.0065
K	0.18	9.0	2.0
Na	95.9	94.0	95.5
P	6.7	14.6	0.095
Zn	0.04	0.095	0.005

Analysis was performed by AgResearch, Palmerston North.

From Table 3.2, it can be seen that iron was present in the original protein solutions in very small amounts (approximately 0.006 mg/g). The zinc content in the WPI solution was also very low (approximately 0.005 mg/g) but the levels in sodium caseinate and

MPC were somewhat higher (0.04 and 0.1 mg/g respectively). These slightly elevated levels of zinc could be due to the presence of casein. It is reported that cow's milk, which has 80% casein, has a significantly higher zinc content (approximately 3.9 mg/L) than human milk (approximately 1.2 mg/L) (Flynn and Cashman, 1997). The concentration range of iron in the binding experiment was 0 to 1120 mg/L (0 to 20 mM) while the range for zinc was 0 to 654 mg/L (0 to 10 mM). These levels are far in excess of the mineral concentrations in the protein solutions.

Very small amounts of copper were found in all protein powders (0.001–0.003 mg/g powder). At these levels, copper ions are unlikely to significantly affect the rate of lipid oxidation of the protein powders during experimentation.

For each protein solution, the amount of sodium was high. This was due to the addition of NaOH when the pH of these solutions was adjusted to 6.6. Calcium and phosphorus contents in MPC were high. This was due to the presence of casein micelles in MPC. Conversely, WPI had relatively small amounts of these two minerals. Sodium caseinate had a low calcium content, but its phosphorus content was high. This was probably due to the presence of organic phosphate (phosphoserine residues) in caseins. MPC had the highest amount of potassium, followed by WPI and sodium caseinate.

3.2. Methodology

3.2.1. Binding of Iron and Zinc to Proteins

3.2.1.1. Preparation of stock solutions

HEPES Buffer, 50 mM, pH 6.6, was prepared by dissolving 11.92 g of HEPES buffer, 5 mL of 1 M NaOH and 95 mL of 1 M NaCl in approximately 800 mL of milliQ water. The pH was adjusted to 6.6 and the solution was made up to 1 L. The buffer had an ionic strength of 0.1 M. A 50 mM stock solution of the mineral (either ferrous sulphate or zinc sulphate) was prepared in this HEPES buffer.

3.2.1.2. Solubility of iron and zinc

The solubilities of ferrous sulphate and zinc sulphate were determined at different concentrations in the absence of proteins. The stock solution (50 mM) was diluted with HEPES buffer to give a final iron or zinc concentration between 0 mM and 20 mM. The pH of the solutions was adjusted to 6.6 using 0.5 M NaOH. The solution was then mixed, held for different times (0 to 2 h) at room temperature and then centrifuged (Model RC5C, Sorvall Instrument, DuPont, USA) for 20 min at 10,800 *g*. The supernatant was collected and analysed for its iron and zinc content. The solubility was measured as the amount of mineral in the supernatant as a percentage of the total.

3.2.1.3. Experimental procedure

20 g of a protein (e.g. sodium caseinate) was dissolved in 1 L of 50 mM HEPES buffer. To 25 mL of this protein solution, either iron or zinc was added from the stock solution and then HEPES buffer was added to give a final volume of 50 mL. This gave a final concentration of 1% protein and a final concentration of iron ranging from 0 to 20 mM or a final concentration of zinc ranging from 0 to 10 mM. This solution had an ionic strength of 0.1 M. The pH of the solution was adjusted to 6.6 using 0.5 M NaOH. The mineral-protein mixtures were shaken and then left at room temperature (approximately 20°C) for 2 h. At this point, the turbidity of the mineral-protein mixtures was measured to determine the extent of aggregation (Section 3.2.3.4). The mixtures were then centrifuged at 10,800 *g* at 20°C, for 20 min. This produced a sediment which contained insoluble protein and insoluble mineral and a supernatant which contained soluble protein and soluble mineral. The supernatant was carefully decanted and analysed for mineral and protein concentrations.

To separate the soluble "free" mineral from the mineral bound to soluble protein, a portion of the supernatant was passed through an Amicon stirred ultrafiltration (UF) cell (Model 8050, Amicon Div., W.R. Grace and Co. Denvers, MA, USA), containing a Diafilo UF membrane YM 10 (MW cut-off 10,000), at a pressure of 300 kPa. The UF permeate, which contained the soluble free mineral, was analysed for its mineral concentration. The summarised experimental procedure is shown in Figure 3.1.

All binding experiments were carried out at least in duplicate. Data presented in the thesis are the average of at least two separate experiments. For selected experiments, statistical analysis was applied to the data using Minitab 14 for Window package. Standard errors are shown in selected figures.

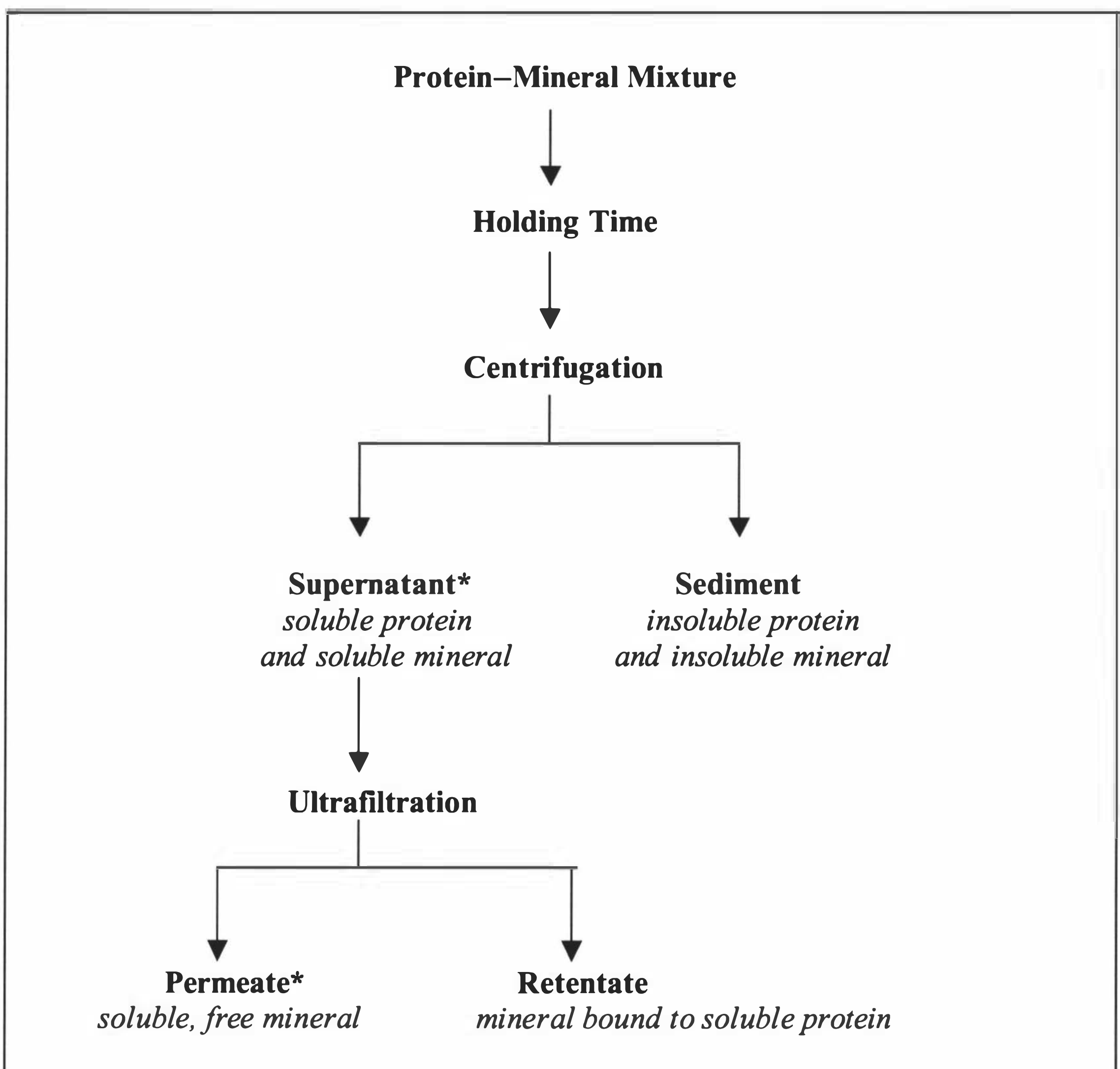


Figure 3.1. Experimental procedure for binding experiments.
* Samples analysed on a routine basis.

3.2.1.4. Binding to individual proteins

For some experiments, the extent of binding of the mineral to individual milk proteins in the samples was investigated. This was done by running supernatant samples on electrophoresis gels (Section 3.2.3.3).

3.2.1.5. Effect of pH

For the binding experiments, the pH of the mineral-protein mixture was normally adjusted to 6.6. However, for some experiments the pH of the 2% protein solution in HEPES buffer was adjusted over the range 2.0 to 7.0 with either 0.5 M HCl or 0.5 M NaOH. This solution was held at 4°C, overnight. Either iron or zinc was added to the protein solution followed by HEPES buffer to give a 1% protein solution. The normal procedure was then followed.

3.2.1.6. Effect of ionic strength

The ionic strength of the mineral-protein mixture for the binding experiments was normally 0.1 M. However, for some experiments, the ionic strength was adjusted. Protein solutions containing a protein concentration of 1% and a mineral concentration at 2 mM were prepared. The ionic strength of these solutions was then adjusted to 0.25 M and 0.5 M by adding NaCl. The normal procedure was then followed.

3.2.1. Mass Balance

A mass balance was performed to determine the extent of recovery of minerals throughout the binding experiment. Samples to be analysed for their mineral content were digested and made up to volume with 2 M HCl. They were measured using atomic absorption spectroscopy (AAS) against standards prepared in 2 M HCl (see Section 3.2.3.2).

The results for one experiment are shown in Figure 3.2. A second experiment was conducted and it produced similar results.

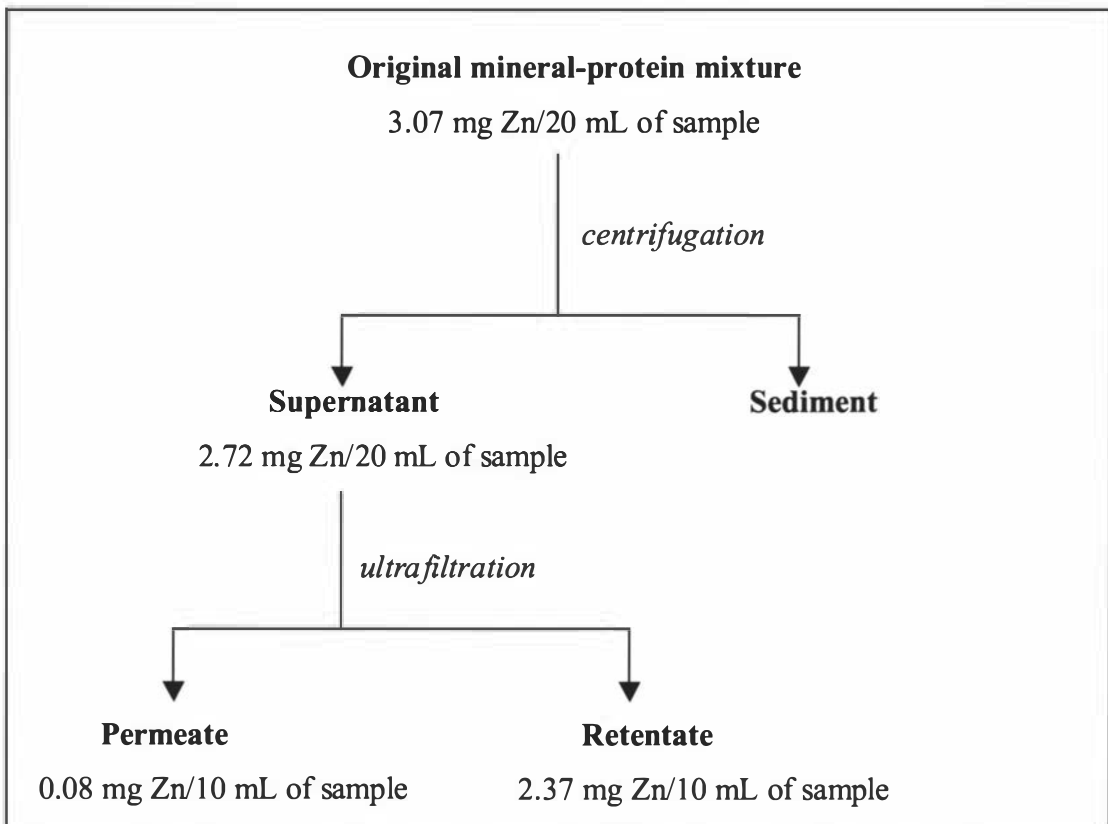


Figure 3.2. Mass balance experiment.

A 1% protein solution containing approximately 2.25 mM zinc, adjusted to pH 6.6, was prepared. This solution was analysed and found to contain 153.5 mg/L zinc. 20 mL of this sample was taken for the mass balance experiment. It was found that after centrifugation, approximately 90% of the zinc present in the original sample was recovered in the supernatant sample. This suggested that about 10% of the zinc was present in the sediment. However, because there was only a very small amount of sediment, it was not possible to recover it for analysis. After ultrafiltration, approximately 90% of the zinc in the supernatant was found in the permeate and retentate. In this instance, the 10% loss of zinc could be due to zinc being loosely bound to the UF membrane. The mass balance showed that there were no major losses of zinc during experimentation.

3.2.3. Analysis of Samples

3.2.3.1. Protein content

The protein content of the samples was measured using the Kjehdahl method (AOAC, 1974). Protein contents were calculated from the nitrogen content by multiplying the total nitrogen content by a factor of 6.38.

3.2.3.2. Mineral content

The mineral contents of the mineral-protein samples were determined by AAS (GBC 933 AA, GBCC Scientific Equipment Pty Ltd, Dendenong, Australia). The acetylene-air flame was optimised prior to the measurement of samples.

Iron and zinc standards were prepared using a certified standard solution (Spectrosol). The standards were normally made up in purified water. For one set of experiments, the standards were made up in a 0.01% sodium caseinate solution. These standards gave the same readings as standards prepared in water, indicating that protein solutions at a concentration of 0.01% did not interfere with AAS measurements (Figure 3.3).

The mineral-protein samples were normally diluted 100 fold prior to analysis. This meant that for all samples measured on a routine basis, the protein concentration was $\leq$ 0.01%. The results for standard solutions (see Figure 3.3) indicate that at this level, the protein does not interfere with the spectroscopic readings.

For the mass balance experiments, the samples (original, supernatant, retentate and permeate) were digested with acid prior to analysis by AAS. The sample was weighed, concentrated nitric acid was added and the sample was left soaking overnight. The sample was then heated at approximately 90°C. Once the nitrous oxide gas has disappeared, the temperature was raised to 120°C to evaporate the sample to near dryness. The sample was then made up to volume with 2 M HCl. These digested samples were diluted 100 fold with 2 M HCl and then measured. The standards for these samples were also prepared in 2 M HCl.

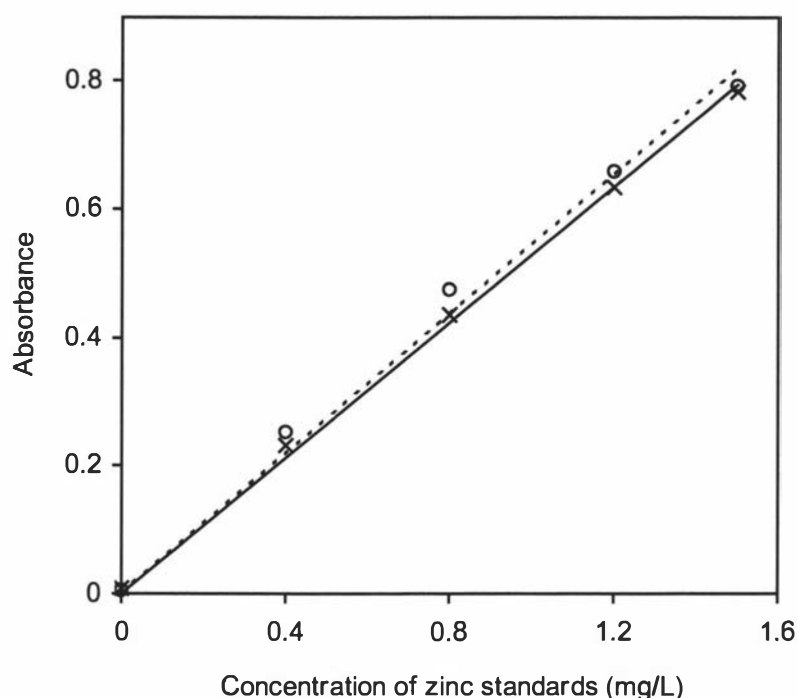


Figure 3.3. Absorbance readings from AAS of zinc standards at varying concentrations. (**o**) Standards dissolved in water. (**x**) Standards dissolved in 0.01% sodium caseinate.

The samples for the mass balance experiments were digested because the retentate sample contained a significant amount of protein (about 2%) which it was thought might interfere with AAS measurements. It should be noted that retentate samples were not measured on a routine basis.

3.2.3.3. Electrophoresis

The compositions of the protein samples were determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were analysed using the Mini-Protean II dual slab cell system (Bio-Rad Laboratories, Hercules, CA 94547, USA). The resolving gel buffer was 1.5 M Tris-HCl buffer, pH 8.8. The stacking gel buffer was 0.5 M Tris-HCl buffer, pH 6.8. Stock acrylamide (30%) was prepared from 30 g acrylamide and 0.8 g N, N-bis acrylamide and then made up to a final volume of 100 mL with deionised water. Sample buffer was prepared by mixing 500 mL of

distilled water, 125 mL of 0.5 M Tris-HCl buffer (pH 6.8), 100 mL of glycerol, 200 mL of 10%(w/v) SDS and 25 mL of 0.05%(w/v) bromophenol blue to give a total volume of 1 L.

The resolving gel (16% w/v, 0.375 M TRIS, pH 8.8) was prepared by mixing 2.02 mL deionised water, 2.5 mL of 1.5 M Tris-HCl buffer, 100 μL of 10% SDS, and 5.3 mL of acrylamide (30%). The resolving gel was degassed for 20 min while stirring and 5 μL of TEMED (N,N,N,'N'-tetramethylethylenediamine) and 50 μL of freshly made ammonium persulphate (10% w/v) were added and mixed gently. The resolving gel was poured between two glass plates and overlaid with deionised water. The resolving gel was then left at room temperature (approximately 20°C) for 45 min to set and the water was removed from the top of the resolving gel prior to application of the stacking gel.

The stacking gel was prepared from 3.05 mL deionised water, 1.25 mL of 0.5 M Tris-HCl buffer, 50 μL of 10% SDS and 0.65 mL acrylamide (30%). After degassing, 5 μL TEMED and 25 μL ammonium persulphate were added and gently mixed. The stacking gel was poured on top on the resolving gel and a 10-slot comb was inserted.

Two of these combined gels (resolving and stacking gels) were placed into an electrode buffer chamber. The stock electrode buffer was made from 9 g of Tris base, 43.2 g glycine and 3 g of SDS, made up to 600 mL with deionised water. After adjusting the pH to 8.3, the volume of the buffer was made up to 1 L. The stock electrode buffer was diluted 1:4 with deionised water and used to fill the inner buffer chamber. The supernatant sample was diluted 10 fold using the sample buffer. A Hamilton syringe (Hamilton Company, Reno, Nevada, USA) was used to inject 10 μL of this sample into a well. Skim milk (approximately 4% protein), diluted 40 fold using sample buffer, was used as a standard. The gels were then run using a power supply from Bio-Rad Model 1000/500 with the maximum voltage, current, power and time set at 210 V, 70 mA, 6.5 W and 1 h, respectively. The gels were run until the tracking dye (bromophenol blue) had run off the bottom of the resolving gel.

Each gel was stained for 1 h in 50 mL brilliant blue staining solution (3 g brilliant blue, 200 mL acetic acid glacial, 500 mL propan-2-ol in 2 L of deionised water) in a closed 500 mL container with continuous agitation. This was followed by two destaining steps of 1 and 19 h respectively, with 100 mL of destaining solution (10% propan-2-ol, 10% acetic acid glacial). The gels were then photographed. Band intensities were quantitatively determined using an Ultrascan XL laser densitometer (LKB Produkter, Stockholm-Bromma 1, Sweden). In the densitometer, the protein bands on the stained gel are scanned with a narrow beam of laser light and the absorbance at 522 nm is plotted as a function of track distance. This gives a graph of individual peaks and table of individual peak areas.

3.2.3.4. Turbidity measurements

The turbidity of each mineral–protein mixture (before centrifugation) was measured using a UV/Visible spectrophotometer (Ultrospec II, Pharmacia LKB). Approximately 3 mL of each sample was placed in a 4–mL plastic cuvette. The absorbance of each sample was measured at 650 nm against approximately 3 mL of 50 mM HEPES buffer as the reference standard.

3.2.4. Oxidation Activity of Mineral–Protein Mixtures

A number of different samples containing proteins and minerals were tested to determine the extent to which they oxidised linoleic acid.

3.2.4.1. Preparation of samples

A linoleic acid emulsion consisting of linoleic acid (0.2804 g), Tween 20 (0.2804 g) and 50 mL phosphate buffer (0.2 M, pH 7.4) was prepared at room temperature using a laboratory high-speed mixer (10,000 rev.min^{-1}) (Heidolph, Diax 600, Germany).

A series of different samples containing proteins and/or minerals were prepared as follows. 1% protein solutions were prepared by dissolving the protein powders in HEPES buffer. Solutions containing free mineral (either iron or zinc) were prepared by

mixing 25 mL of 50 mM HEPES buffer and either iron or zinc from the stock solution. HEPES buffer was then added to give a final volume of 50 mL and a final mineral concentration of 1 mM. Mineral–protein complexes containing a 1 mM final concentration of iron or zinc were prepared by mixing 25 mL of 2% protein solution and either iron or zinc from the stock solution. HEPES buffer was then added to give a final volume of 50 mL. All these sample solutions were then stirred for 2 h at ambient temperature.

After stirring, 1 mL of each of these sample solutions was added to 5 mL of linoleic acid emulsion. These mixtures were then held, without stirring, at 30°C for different times up to 72 h. At the end of the designated time period, samples were analysed for oxidation of linoleic acid. All samples were held in the dark to prevent any oxidation due to light. Control samples of linoleic acid emulsion, held at 4°C and at 30°C in the dark, were also included in the experiment.

3.2.4.2. Thiobarbituric acid (TBA) test

The extent of oxidation of linoleic acid that was caused by the different samples was measured by the thiobarbituric acid (TBA) method according to Hegenauer *et al* (1979a).

TBA Reagent

The TBA reagent was prepared immediately before use by mixing equal volumes of freshly prepared 0.025 M TBA (brought into solution by neutralising with NaOH) and 2 M H_3PO_4/2 M Citric Acid. The combination of citric acid and phosphoric acid was used as both acidulants and metal chelators (Dunkley and Jennings, 1951; Hegenauer *et al*, 1979a).

Assay method

At the end of the holding period, 5 mL of the sample was pipetted into a 50-mL centrifuge tube containing 2.5 mL of the TBA reagent. The contents of the tube were mixed and the tube was placed without delay in a boiling water bath for exactly 10 min.

Heating produced a red-coloured complex formed between malondialdehyde (MDA), a product of the oxidation reaction, and TBA. After heating, the mixture was cooled in ice and 5 mL of cyclohexanone and 1 mL of 4 M ammonium sulphate were then added (in fume hood). Ammonium sulphate was used to desorb the red-coloured compound from the precipitated protein. The red compound then dissolved into the cyclohexanone. The tube was shaken for 2 min, and centrifuged at 6000 *g* for 5 min at room temperature. The orange-red cyclohexanone supernatant was decanted and its absorbance of 532 nm was measured spectrophotometrically (UV–Visible Spectrophotometer, Shimadzu, UV–160A).

It was found that when the orange–red cyclohexanone solution was scanned from 400 to 600 nm, two absorption peaks were observed, one for a yellow–coloured compound at 450 nm and the other for the red-coloured compound at 532 nm (Figure 3.4). There appeared to be very little interference between the two peaks and hence, routine measurements were carried out at 532 nm.

3.2.4.3. Standards for TBA method

Malondialdehyde (MDA) is unstable and standards are therefore usually prepared by acid hydrolysis of either 1,1,3,3-tetraethoxypropane (TEP) or 1,1,3,3-tetramethoxypropane (TMP) (Gutteridge, 1975; Inoue *et al*, 1998). TEP is more suitable because the more polar compound TMP is considerably more resistant to hydrolysis, requiring some four times longer to accomplish complete conversion to MDA (Gutteridge, 1975).

MDA standards were prepared using the method of Botsoglou *et al* (1994). 73.2 mg of TEP was accurately weighed into a screw-capped test tube, dissolved in 10 mL of 0.1 N HCl, immersed into a boiling water bath for 5 min, and quickly cooled under tap water. A stock solution of MDA (239 μg/mL) was prepared by transferring the hydrolysed TEP solution into a 100 mL volumetric flask and diluting to volume with water. The stock solution was diluted further with water to give MDA solutions of various concentrations (0 to 2.2 μg/mL). 5 mL of each of these solutions was reacted with TBA

(as described above) and the red-coloured complex was measured at 532 nm. These results show a linear relationship between absorbance and concentration of the MDA standards (Figure 3.5).

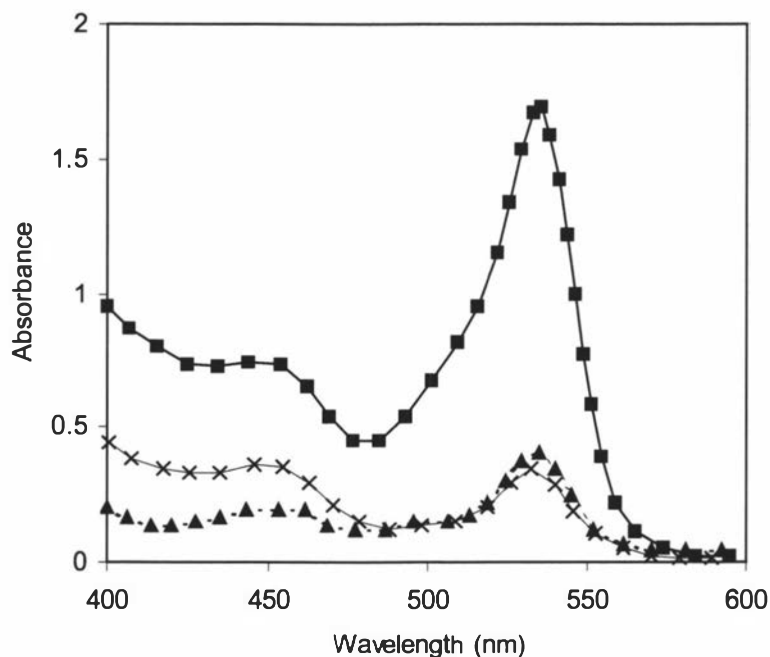


Figure 3.4. Absorption spectra (400 to 600 nm) of TBA reaction products from oxidised linoleic acid. Samples were held at 30°C, in the dark, for 72 hours. (▲) Linoleic acid with no additives. (■) Linoleic acid with free ferrous ions. (x) Linoleic acid with iron-sodium caseinate mixture.

In one experiment, MDA standards were made up in a 1% sodium caseinate solution and reacted with TBA using the procedure described above. The absorbance readings at 532 nm were measured and compared with absorbance readings from standards prepared in water. The two sets of results are virtually the same (Figure 3.5). This suggests that small quantities of dissolved proteins in samples have no effect on the absorbance readings.

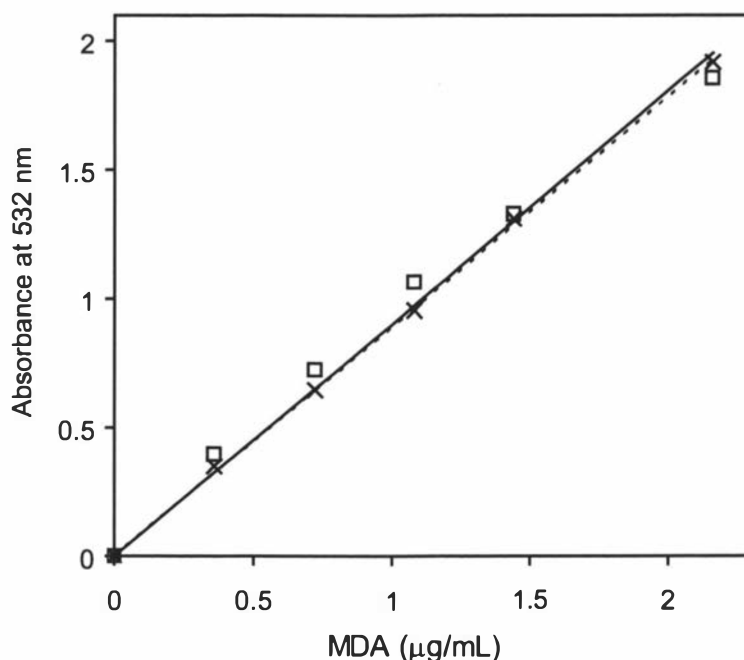


Figure 3.5. Absorbance readings of TBA reaction products of MDA standards (TBA method of Hegenauer *et al*, 1979a). (**x**) Standards prepared in water. (□) Standards prepared in 1% sodium caseinate.

3.2.4.4. Alternative TBA Test

In a further set of experiments, the TBA method of Yen *et al* (1999) was investigated as this method appeared to be a more convenient and rapid procedure than the method of Hegenauer *et al* (1979a).

For the method of Yen *et al* (1999), MDA standards of different concentrations (dissolved in water and in 1% sodium caseinate solution) were prepared as outlined above. 5 mL of a MDA standard was mixed with 2 mL of 0.5% TBA reagent (in water) and 1 mL of 10% HCl. The mixture was heated in a waterbath (approximately 100°C) for 20 min and then cooled. 5 mL of chloroform was added; the mixture was then stirred and centrifuged at 1000 *g* for 20 min. The supernatant (aqueous phase) was decanted and the absorbance at 532 nm was read.

The absorbance readings of these standard solutions are shown in Figure 3.6. It was found that standards dissolved in a 1% sodium caseinate solution gave markedly lower absorbance readings than standards dissolved in water. It can be postulated that most of the red-coloured compound that was formed was absorbed by the sodium caseinate (ammonium sulphate was not present in the mixture to desorb the red compound). Therefore, the TBA method of Yen *et al* (1999) cannot be used in this case as it does not give satisfactory results with samples containing milk proteins.

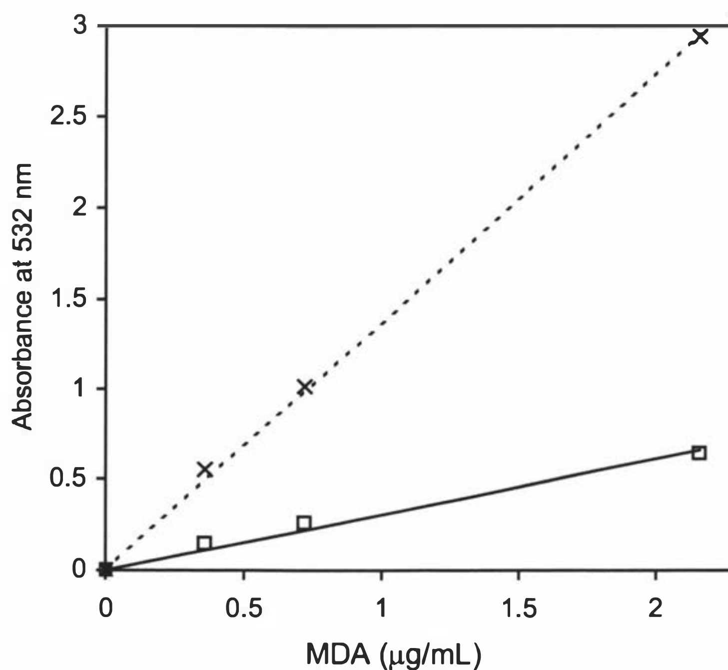


Figure 3.6. Absorbance readings of TBA reaction products of MDA standards (TBA method of Yen *et al*, 1999). (**x**) Standards prepared in water. (□) Standards prepared in 1% sodium caseinate.

CHAPTER 4
BINDING OF IRON TO MILK PROTEIN PRODUCTS

4.1. Introduction

Iron is considered to be one of the essential minerals required by the human body. Although milk is a good source of minerals, its iron content is too low (0.2–0.5 mg iron/L) to meet daily dietary requirements (Flynn and Cashman, 1997). Therefore, incorporating iron into milk or dairy products has been considered as a potential approach to prevent iron deficiency in humans, which is a major nutritional problem worldwide (Hurrell and Cook, 1990). When iron is added to milk or milk products, binding of the iron to the milk proteins occurs. The mineral–protein complexes that are formed are relatively stable to oxidation and processing conditions and are organoleptically acceptable.

Much research has focused on adding iron to milk or milk products, either for fortification or to enhance the amount of iron bound to various milk components. Iron has been added to raw milk (Demott and Dincer, 1976; Hegenauer *et al*, 1979a; Reddy and Mahoney, 1991a), skim milk (Gaucheron *et al*, 1997), pasteurised skim milk (Carmichael *et al*, 1975; Hegenauer *et al*, 1979a; Hekmat and McMahon, 1998) and whole milk (Hegenauer *et al*, 1979a). Iron has also been added to individual casein fractions (α_s-, β- or κ-casein) (Manson and Cannon, 1978; Baumy and Brule, 1988b; Reddy and Mahoney, 1991b) and to sodium caseinate (Nelson and Potter, 1979; Gaucheron *et al*, 1996, 1997a).

Furthermore, iron has been shown to complex with whey proteins. Jones *et al* (1972, 1975) showed that iron polyphosphate–protein powder could be made from cheese whey through a precipitation technique using 'ferripolyphosphate', which was a soluble complex of ferric ions with a long chain phosphate. Addition of ferripolyphosphate to whey at pH 4.6 caused precipitation and the formation of ferripolyphosphate–whey

protein, which contained 12–15% iron. Amantea *et al* (1974) produced a soluble whey protein–ferric ion complex containing greater than 80% protein. Another iron–whey protein complex was ferrilactin, which was a complex formed between ferric iron and whey proteins under acidic conditions, as reported by Jackson and Lee (1992).

Most of the iron that is added to milk is bound to the casein fractions and only a small percentage is bound to the whey protein fractions (Carmichael *et al*, 1975; Hegenauer *et al*, 1979a). The strong affinity of the caseins for iron has been recognised and is attributable mainly to clustered phosphoserine residues in the phosphoproteins (Demott and Dincer, 1976; Manson and Cannon, 1978; Hegenauer *et al*, 1979a; Gaucheron *et al*, 1996, 1997a). The relative affinities of individual milk proteins for iron (Fe^{3+}) has been shown by Vaughan and Knauff (1961) to be: α_{s1}-casein > β-casein > BSA > κ-casein > β-Lg > α-La.

The process of iron binding to milk proteins is affected by different conditions such as pH, ionic strength, holding time and temperature. The differences in the protein structures and the binding abilities of the different milk proteins result in different binding characteristics. This binding also affects the solubility, and changes the protein structure.

The present study focused on determining systematically the binding of iron by commercial milk protein products. The objectives of this part of the work were to characterise the binding of iron to sodium caseinate, whey protein isolate (WPI) and milk protein concentrate (MPC), and to determine how this binding is affected by different environmental factors.

4.2. Solubility of Ferrous Sulphate in HEPES buffer

The solubility of ferrous sulphate ($FeSO_4.7H_2O$) in 50 mM HEPES buffer (pH 6.6) was investigated by mixing various amounts of ferrous sulphate in the buffer, leaving for 2 h at room temperature, and then centrifuging at 10,800 *g* for 20 min. The solubility was

measured as the amount of iron in the supernatant as a percentage of the total amount of added iron.

The solubility of iron in HEPES buffer was found to be very low at low iron concentrations (Figure 4.1). Only about 10% of the total iron was found in the supernatant when up to 2 mM iron was dissolved in HEPES buffer. The solubility increased gradually at > 3 mM added iron and approximately 75% was found to be soluble at 20 mM added iron.

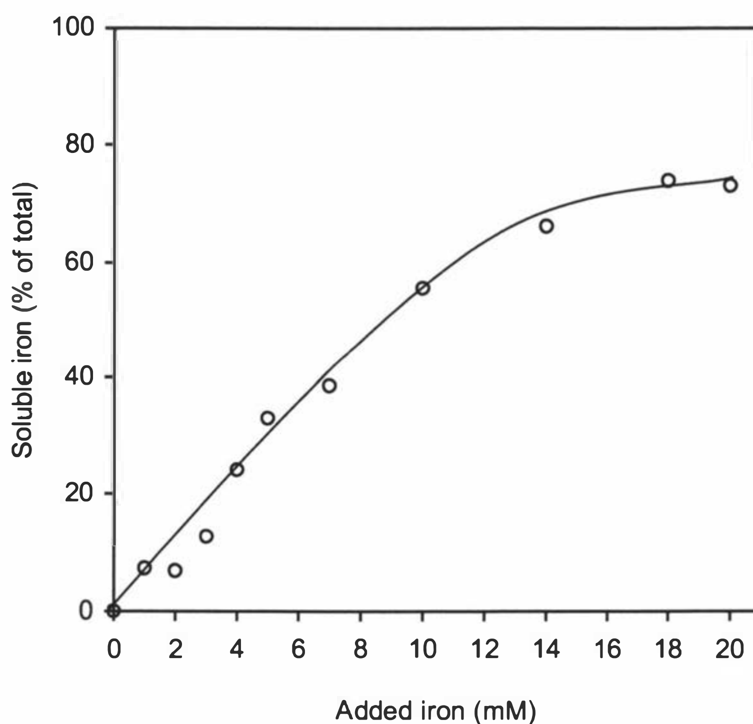


Figure 4.1. Solubility of ferrous sulphate in 50 mM HEPES buffer at pH 6.6.

The low solubility of the ferrous iron under the experimental conditions may have been due to a number of factors. Ferrous salt is reported to be more stable in acidic aqueous solutions (Spiro and Saltman, 1974). In solutions of low pH, ferrous iron exists in a hydrated form (Clydesdale, 1983). As the pH is raised to neutral or alkaline, protons are lost and a hydroxide is formed. This hydroxide is considerably less soluble than the

hydrate and will precipitate out of the solution. Hydroxides precipitate from solutions of Fe^{2+} at above pH 5.5 and from solutions of Fe^{3+} at above pH 2.0–3.0 (Eyerman *et al*, 1987). Therefore, it is likely that, under the experimental conditions, the iron was insoluble because of the formation of the insoluble iron hydroxide at pH 6.6.

However, an investigation by Nelson and Potter (1989) gave a different result from that of the present study. Nelson and Potter (1989) studied the solubility of ferrous sulphate ($FeSO_4.H_2O$) by dissolving 20 mg Fe in 50 mL deionised water (approximately 7.1 mM) and adjusting the pH to 4, 5, 6, 7, 8 or 10. They found that, under these conditions, ferrous sulphate was completely soluble at pH 4 to pH 6.

The different results obtained in these two studies may have been due to the different holding times for the ferrous sulphate solution before centrifugation. In the present study, the solutions were kept for 2 h at room temperature before centrifugation whereas Nelson and Potter (1989) centrifuged the ferrous sulphate solution immediately after mixing. During the 2-h holding period at pH 6.6, the insoluble ferric hydroxide could form because it is known that ferrous iron readily oxidises to ferric iron in the presence of oxygen (Aslamkhan *et al*, 2002). An attempt was made to suppress the presence of oxygen in the environment by flushing with nitrogen gas. When the solution was flushed with nitrogen, the solubility of the ferrous sulphate (under the same conditions) was only slightly improved (Figure 4.2), probably because the water still contained dissolved oxygen after flushing.

It is evident that the pH of a solution has a marked effect on Fe^{2+} oxidation. The typical half-life ($t_{½}$) of Fe^{2+} oxidation (with a partial pressure of 0.2 atm of O_2 at 25°C) has been reported to decrease dramatically from 5–6 years at below pH 3.5 to only 4 min at pH 7.0. (Aslamkhan *et al*, 2002). In view of the importance of pH, the solubility of ferrous sulphate (2 mM) in 50 mM HEPES buffer was investigated at pH 3.0, 5.0 and 6.6, with the samples centrifuged at different time intervals up to 2 h. The results showed that, at pH 5.0 and 3.0, most (> 90%) of the ferrous sulphate was soluble even after 2 h (Figure 4.3). However, at pH 6.6, only about 50% of the ferrous sulphate was

soluble at 0 h and only about 30% was soluble after 2 h. These results confirm that, at pH 6.6, ferrous sulphate is less soluble than at acid pH (e.g. pH 3.0 and 5.0) and that, at longer holding time, in the presence of air, the solubility decreases further. This result is also in agreement with the results obtained by Eyerman *et al* (1987), who studied the degree of hydrolysis of different iron sources (one of which was ferrous sulphate) in model systems using the Prep Tyrode buffer at the pH of the endogenous model system. They found that ferrous sulphate at a concentration of 1.4 mM (77.5 ppm) was only about 33% soluble (the solubility was determined from the supernatant after centrifugation at 2335 *g* for 10 min) and that this soluble iron was found to decrease in a linear manner with time.

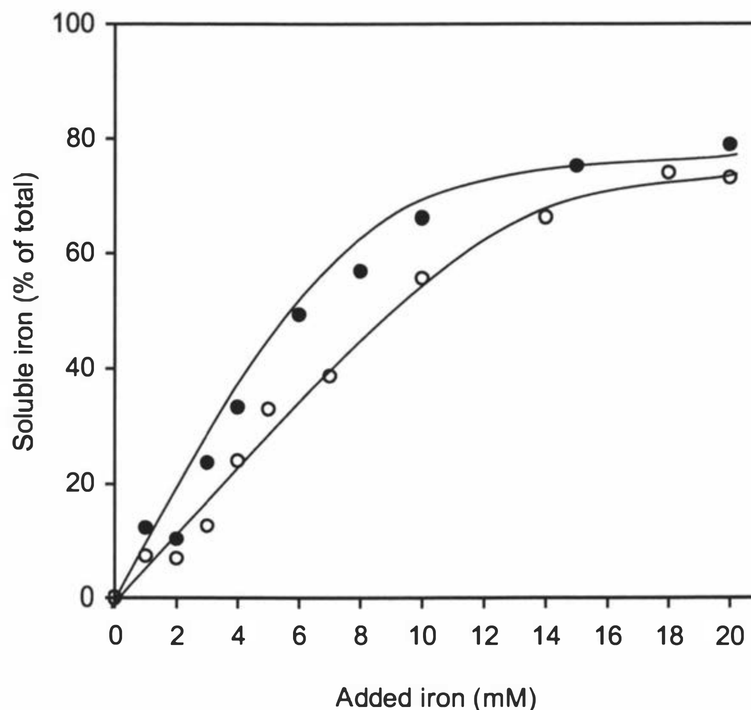


Figure 4.2. Solubility of ferrous sulphate in the presence of oxygen (o) and in a nitrogen-flushed environment (•), in 50 mM HEPES buffer at pH 6.6.

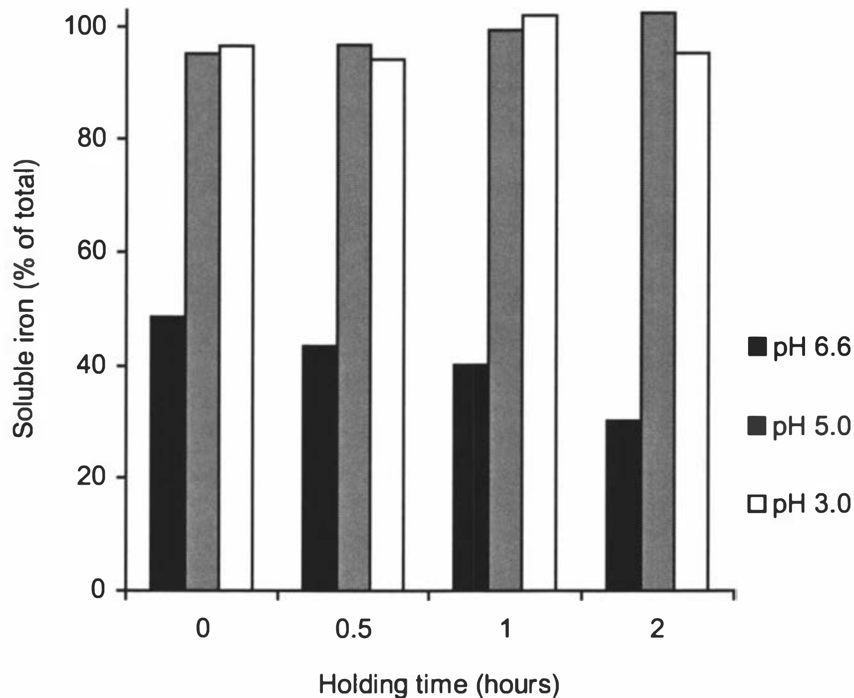


Figure 4.3. Solubility of ferrous sulphate at different pH and holding times, in 50 mM HEPES buffer. The iron concentration used was 2 mM.

4.3. Solubility of Iron and Sodium Caseinate in Iron–Sodium Caseinate Mixtures

The solubility of iron in the presence of sodium caseinate was markedly different from its solubility without addition of the protein (Figure 4.4). For the range from 0 to 4 mM added iron, more than 90% of the iron was found in the supernatant, when the solution contained 1% sodium caseinate. Hence, for this concentration range, the presence of sodium caseinate markedly improved the solubility of iron. Between 4 and 8 mM added iron, the solubility of iron decreased markedly, but there was a slight increase in solubility with increasing iron concentration above 8 mM.

The solubility of sodium caseinate in the presence of iron was constant at approximately 90% up to 4 mM added iron (Figure 4.5). Above this concentration, addition of iron

caused precipitation and subsequent sedimentation of sodium caseinate. The solubility of sodium caseinate decreased linearly with the addition of iron (> 4 mM) and the solubility was < 10% at 20 mM added iron.

Precipitation of caseins caused by the addition of iron has been reported previously. Baumy and Brule (1988b) found that a minimum concentration of ferrous chloride of 7 moles/mole of protein was required to precipitate β-casein. Reddy and Mahoney (1991a) reported that binding of Fe^{3+} to bovine α_{s1}-casein led to precipitation of the protein, and Nelson and Potter (1979) observed a precipitation of protein when ferrous sulphate (20 mg Fe/g protein) was added to sodium caseinate.

In a later study, Gaucheron *et al* (1996) showed that a sodium caseinate solution (25 g/L) remained unaffected by the addition of up to 4 mM added ferrous chloride (approximately 9 mg Fe/g protein), but casein was completely insoluble at an iron concentration of 7.5 mM. In the present study, the precipitation of the sodium caseinate solution (10 g/L) was found to increase gradually on addition of iron above 4 mM (approximately 22 mg Fe/g protein) (Figure 4.5). Hence, in the present study, it appeared that a greater amount of iron was required to precipitate the sodium caseinate.

The disagreement between these two studies may have been due to the difference in pH of the iron–sodium caseinate mixtures. In the present study, the pH of the iron–caseinate mixture was adjusted to 6.6 after addition of the iron, whereas Gaucheron *et al* (1996) did not adjust the pH. When the pH was not adjusted, addition of iron to the sodium caseinate solution caused a decrease in pH. The pH of the sodium caseinate solution (without iron added) was 6.62 and decreased to 6.5 at a final iron concentration of 1.5 mM (3.4 mg Fe/g protein) (Gaucheron *et al*, 1996). Adjustment of the pH from 6.5 to 6.6 means that the protein molecules are more negatively charged and hence more positively charged iron is required to cause aggregation and precipitation.

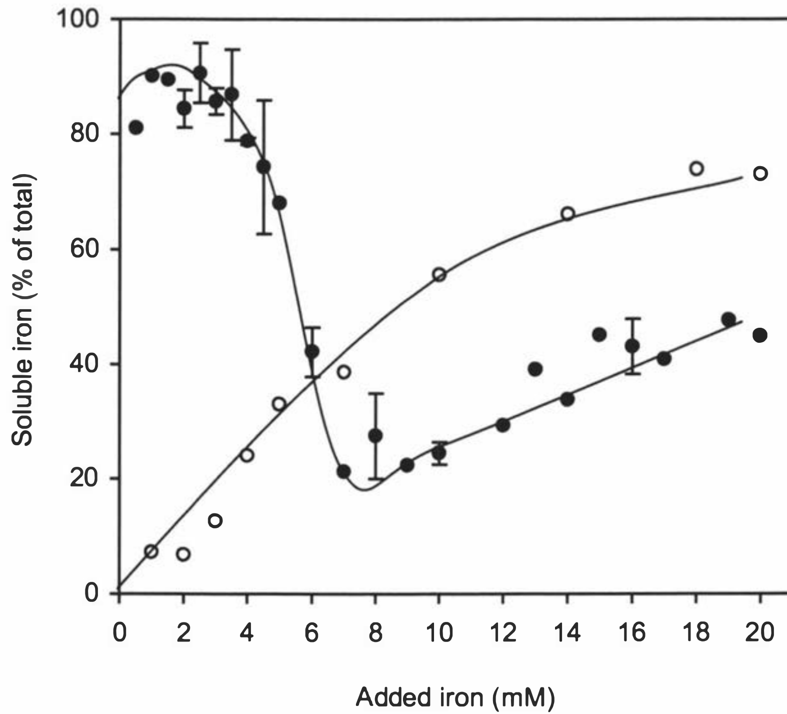


Figure 4.4. Solubility of iron in the absence (o) and in the presence (•) of sodium caseinate, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

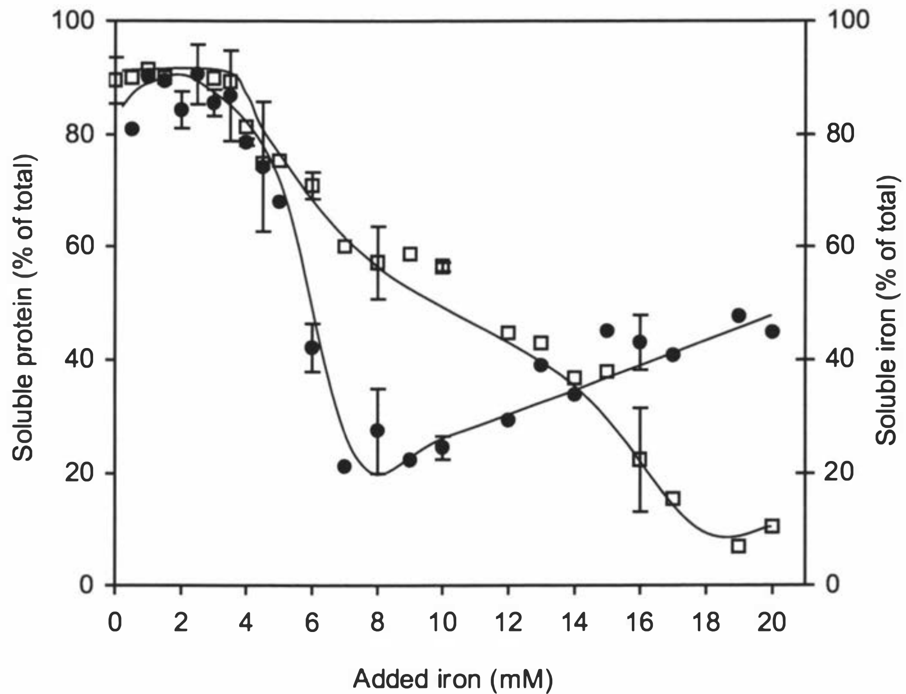


Figure 4.5. Solubility of iron (•) and sodium caseinate (□) in iron–sodium caseinate mixtures, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

In order to confirm the above hypothesis, the effect of pH (5.2 to 7.0) on the aggregation of iron–sodium caseinate mixtures was investigated (Figure 4.6) at different iron concentrations. The extent of aggregation was determined using turbidity measurements at a wavelength of 650 nm. The results showed that: (i) the turbidity increased as the amount of added iron increased, (ii) the increase in turbidity became more rapid when the pH of the mixtures was decreased and (iii) the turbidity versus iron concentration curve shifted towards a lower iron concentration with the pH decrease. These findings suggest that, when the pH of the mixture is high, more Fe^{2+} is required to associate with the negative charges on the protein to initiate aggregation.

Analysis of the iron content of the supernatant after the aggregates in the iron–sodium caseinate mixtures were centrifuged showed that the solubility of the iron was affected when the mixtures were adjusted to different pH values (Figure 4.7). As shown by the turbidity data, the decrease in pH (from 7.0 to 5.2) caused a shift in the critical amount of iron that had to be added to the sodium caseinate before the iron became insoluble. This critical amount of added iron that was required to precipitate the iron from the iron–sodium caseinate mixtures was > 5.0, 3.5, 2.5 and 1.5 mM at pH 7.0, 6.6, 6.2 and 5.8 respectively and was < 0.5 mM Fe at pH 5.2 (Figure 4.7). This precipitation of iron from iron–sodium caseinate mixtures was probably due to the aggregation of sodium caseinate as the pH was decreased towards its isoelectric point (pH 4.6).

Above these minimum concentrations, the solubility of iron in the mixtures decreased markedly before it started to increase slightly with increasing amounts of added iron. This increase was more marked at low pH (e.g. pH 5.2) than at high pH (e.g. pH 7.0) (Figure 4.7). At higher concentrations of added iron (e.g. 9 mM), it was found that the sodium caseinate was approximately 90% insoluble at all pH. Because the caseinate was insoluble, the solubility behaviour of the iron was similar to that in the absence of protein. Iron was found to be more soluble at low pH in the absence of protein (Figure 4.3). Hence, it is likely that there was an increase in the solubility of iron at higher concentrations of added iron (above the minimum concentrations mentioned above).

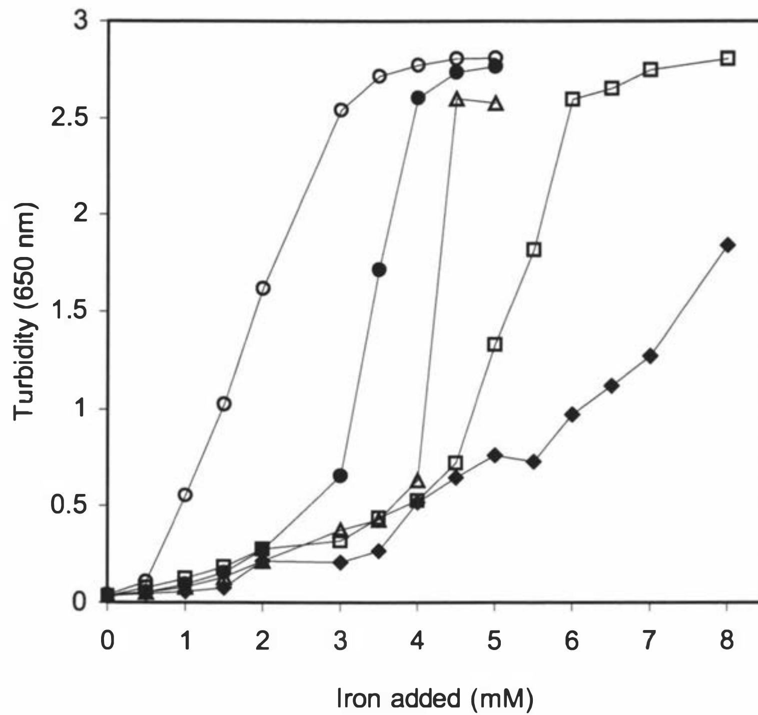


Figure 4.6. Effect of pH: (♦) pH 7; (□) pH 6.6; (△) pH 6.2; (•) pH 5.8 and (o) pH 5.2 on the turbidity (650 nm) of iron–sodium caseinate mixtures, in 50 mM HEPES buffer.

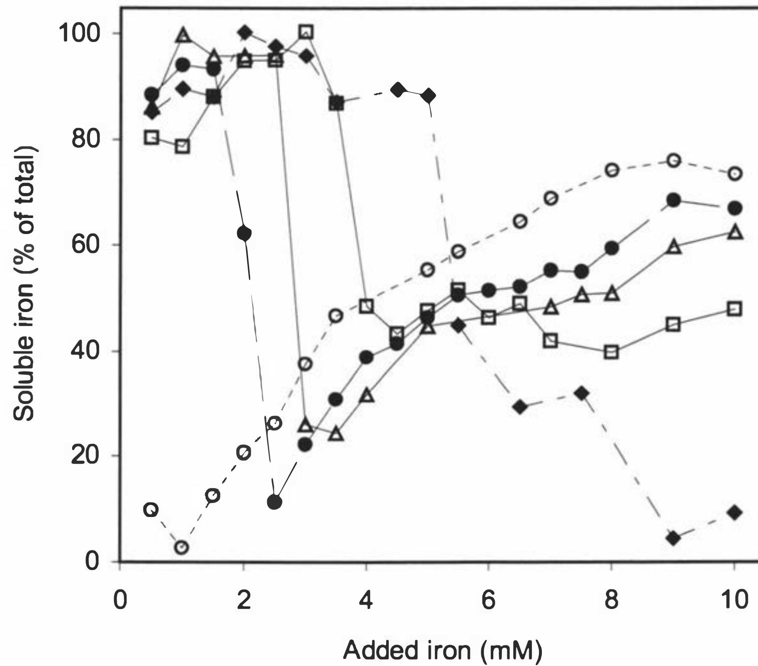


Figure 4.7. Effect of pH: (♦) pH 7; (□) pH 6.6; (△) pH 6.2; (•) pH 5.8 and (o) pH 5.2 on the solubility of iron in iron–sodium caseinate mixtures, in 50 mM HEPES buffer.

It was also found that the extent of aggregation of the protein upon addition of iron was time dependent (Figure 4.8). At a given time, the turbidity increased as the amount of iron added increased; the turbidity also increased with holding time. The increase in turbidity was more marked at added iron concentrations of 4 and 8 mM. At an added iron concentration of 20 mM, the mixture showed visual precipitation at > 7 h, even before centrifugation.

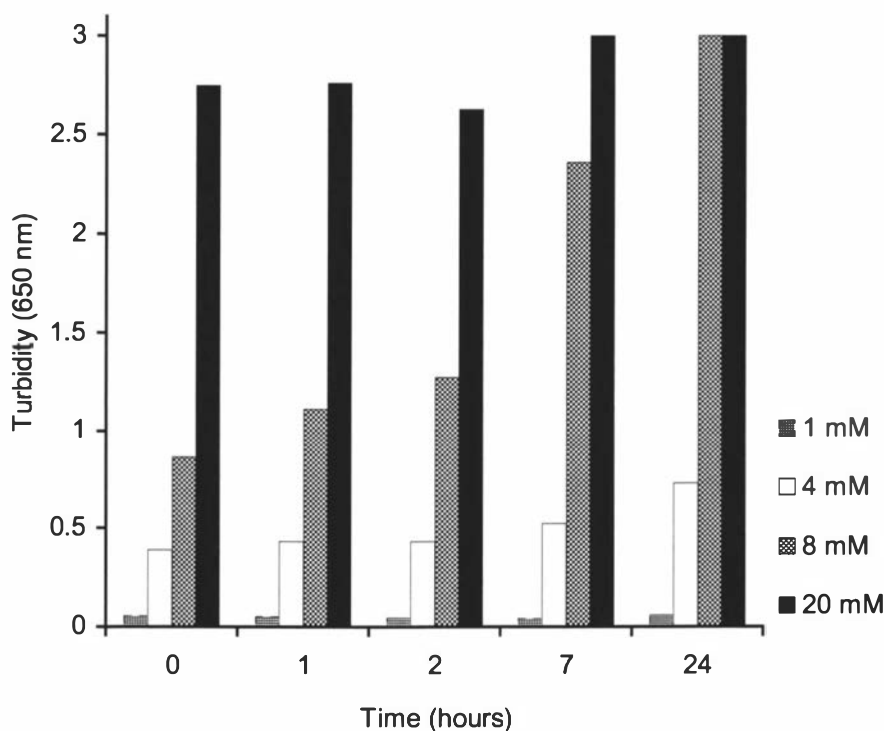


Figure 4.8. Turbidity of iron–sodium caseinate mixtures at different iron concentrations and different holding times, in 50 mM HEPES buffer at pH 6.6.

A qualitative study using potassium ferricyanide ($K_3Fe(CN)_6$) and potassium ferrocyanide ($K_4Fe(CN)_6$) assays was conducted to give an insight into the complexation occurring when iron was added to sodium caseinate (Shears *et al*, 1987). Addition of ferrous iron to potassium ferricyanide produces a dark blue (Turnbulls blue) precipitate, and addition to potassium ferrocyanide produces a light blue precipitate. Addition of ferric iron to potassium ferricyanide produces a brown solution with no

precipitate, and addition to potassium ferrocyanide produces a dark blue (Prussian blue) precipitate. The iron cyanides do not react if the iron is bound strongly (e.g. iron hydroxides).

In the present study, supernatant samples of ferrous sulphate solution and iron–sodium caseinate mixtures at different concentrations of added iron were reacted with the cyanides (Table 4.1). When the supernatant of the ferrous sulphate sample (in HEPES buffer) was reacted with the cyanides, no colour reaction products were produced for the sample containing 1 mM iron and only small amounts of coloured compounds were produced at 4 mM iron. This shows that hardly any Fe^{2+} was left in the supernatant. It was likely that the Fe^{2+} had formed iron hydroxide and had precipitated (Section 4.2) and thus there was very low solubility of the iron in the absence of protein (Figure 4.1). At 8 and 20 mM iron, the presence of Fe^{2+} was detected in the supernatant because > 40% of the iron was soluble at these concentrations (Figure 4.1).

When iron–sodium caseinate mixtures containing 1 and 4 mM iron were reacted with the cyanides (Table 4.1), no coloured reaction products were produced. This indicates that no free Fe^{2+} was present in the samples. No coloured products were produced in the iron–sodium caseinate mixture at 8 mM added iron but the presence of Fe^{2+} was detected at 20 mM added iron. Therefore, it is likely that, at a concentration of up to 4 mM added iron, the caseinate was able to bind all the added iron because the presence of Fe^{2+} was not detected even though approximately 90% of the added iron was soluble (Figure 4.5). At concentrations above 4 mM, the binding of iron by the caseinate continued although not all of the added iron was bound to the protein at 20 mM added iron.

Table 4.1. Qualitative analysis of ferrous sulphate and a mixture of ferrous sulphate and sodium caseinate, dissolved in HEPES buffer, pH 6.6

Iron concentration (mM)	$FeSO_4$	$FeSO_4$ + Sodium caseinate
1	No colour reaction	No colour reaction
4	Traces of Fe^{2+}	No colour reaction
8	Presence of Fe^{2+}	No colour reaction
20	Presence of Fe^{2+}	Presence of Fe^{2+}

4.3.1. Solubility of Individual Caseins in Iron–Sodium Caseinate Mixtures

Mixtures containing 1% caseinate and various levels of ferrous sulphate were centrifuged and the supernatants were analysed using SDS-PAGE.

The results showed that, on addition of iron up to a concentration of 4 mM, the band intensities of β- and κ-caseins were not significantly affected (Figure 4.9), but the band intensity of α_s-casein (α_{s1}- and α_{s2}-caseins) appeared to decrease (Figure 4.10). At ≥ 4 mM added iron, the band intensities of all caseins decreased gradually with an increase in iron concentration, with α_s-casein being affected the most followed by β- and κ-caseins. At ≥ 18 mM added iron, the band intensity of all caseins was low. Thus, it appears that the binding of iron in sodium caseinate affects the solubility of α_s-casein to a greater extent than the solubility of either β- or κ-casein.

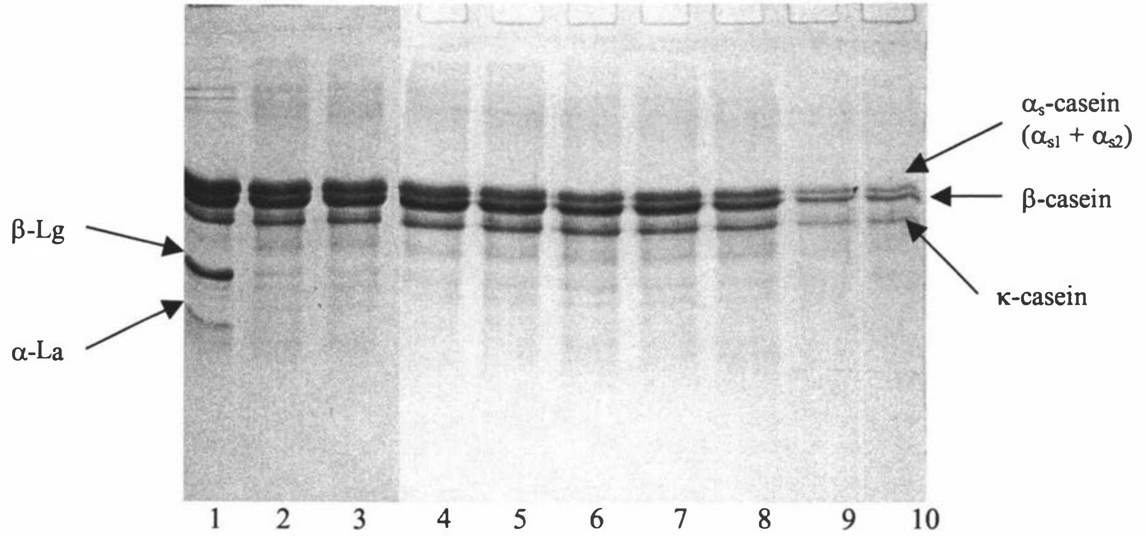


Figure 4.9. SDS-PAGE pattern of: skim milk (1), sodium caseinate solution without addition of iron before centrifugation (2), supernatant obtained after centrifugation (3), and iron–sodium caseinate mixtures after centrifugation with addition of iron at 1 mM (4), 3 mM (5), 4 mM (6), 6 mM (7), 10 mM (8), 18 mM (9), 20 mM (10). Samples were dissolved in 50 mM HEPES buffer at pH 6.6.

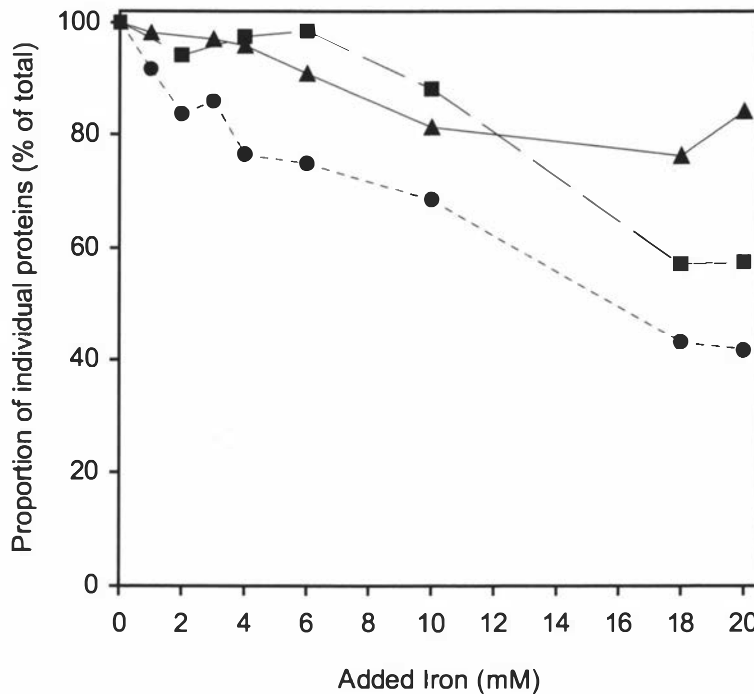


Figure 4.10. Proportions of individual proteins in the supernatant of sodium caseinate solutions: α_s- (α_{s1}- + α_{s2}-) casein (•), β-casein (■) and κ-casein (▲) as affected by the addition of ferrous sulphate, in 50 mM HEPES buffer at pH 6.6.

4.4. Binding of Iron to Sodium Caseinate

Because of the precipitation of sodium caseinate upon addition of iron, the iron bound to sodium caseinate could be separated into two fractions.

- Iron that remained in the supernatant after centrifugation and was retained in the retentate during ultrafiltration. This fraction was bound to soluble protein (referred to as *iron bound to soluble protein*).
- Iron that sedimented along with the protein during centrifugation (referred to as *iron bound to insoluble protein*).

The proportions of iron in both these fractions were calculated based on the *total bound iron*, which is the difference between the total amount of added iron and the amount of free iron in the ultrafiltration permeate. An outline of the distribution of iron in the different fractions of the sample is shown in Figure 4.11. The distribution of total bound iron between the soluble and insoluble fractions of protein is given in Figure 4.12.

Addition of iron at up to approximately 4 mM to sodium caseinate solution resulted in about 90% of the total iron being bound to the soluble protein fraction (Figure 4.12). With increasing amounts of added iron, the relative proportions of iron bound to the soluble protein decreased. At ≥ 8 mM added iron, approximately 20% of the total iron was bound to soluble protein and the remainder was associated with the insoluble fraction of the protein. The decreasing amount of soluble bound iron was caused by the precipitation of sodium caseinate after the addition of > 4 mM iron as shown in Figure 4.5.

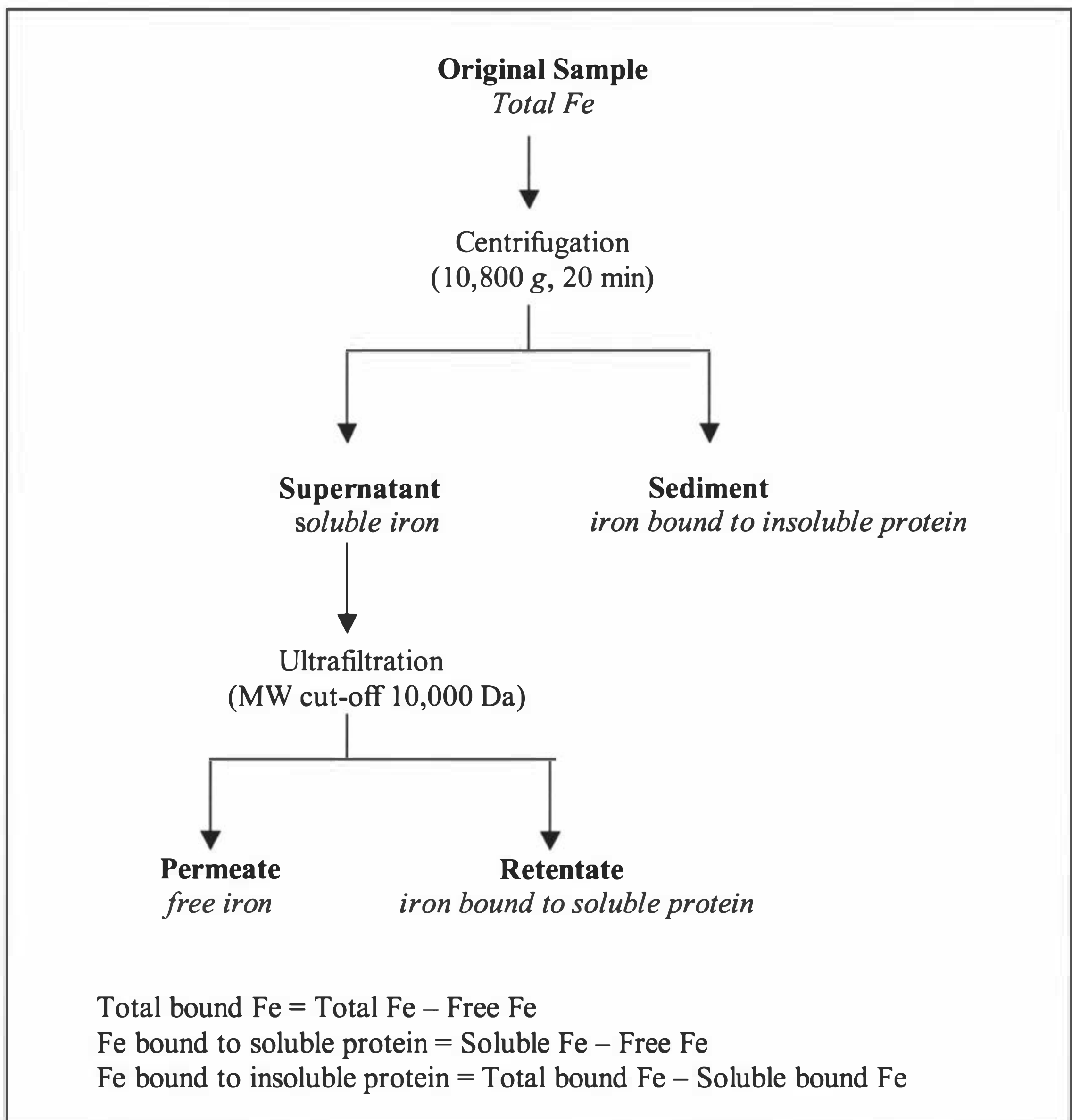


Figure 4.11. Outline of distribution of iron in the different fractions of the sample.

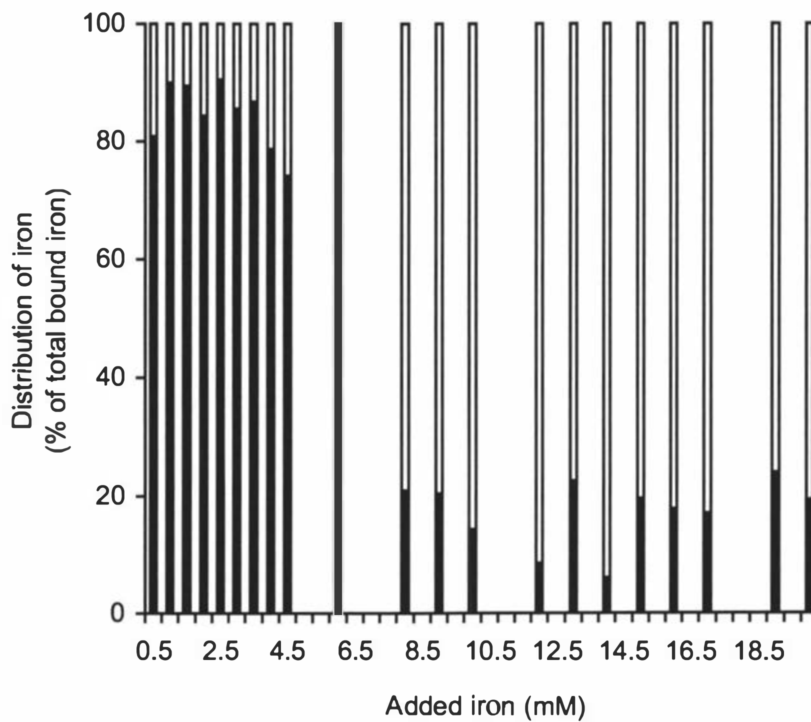


Figure 4.12. Distribution of bound iron (as a proportion of the total bound iron) between the soluble fraction (■) and the insoluble fraction (□) of sodium caseinate dissolved in 50 mM HEPES buffer at pH 6.6.

4.4.1. Binding of Iron to Sodium Caseinate in the Soluble Fraction

In the region where sodium caseinate was still largely soluble (≤ 4 mM iron added), it was found that the concentration of iron in the ultrafiltration permeate (i.e. the free iron) was nearly zero (Figure 4.13). In this region, it was also found that the presence of sodium caseinate markedly increased the solubility of the added iron, from approximately 20% to > 90% (Figure 4.4). Hence, it can be deduced that sodium caseinate bound nearly all the iron added as ferrous sulphate and kept it soluble in the iron–sodium caseinate mixture.

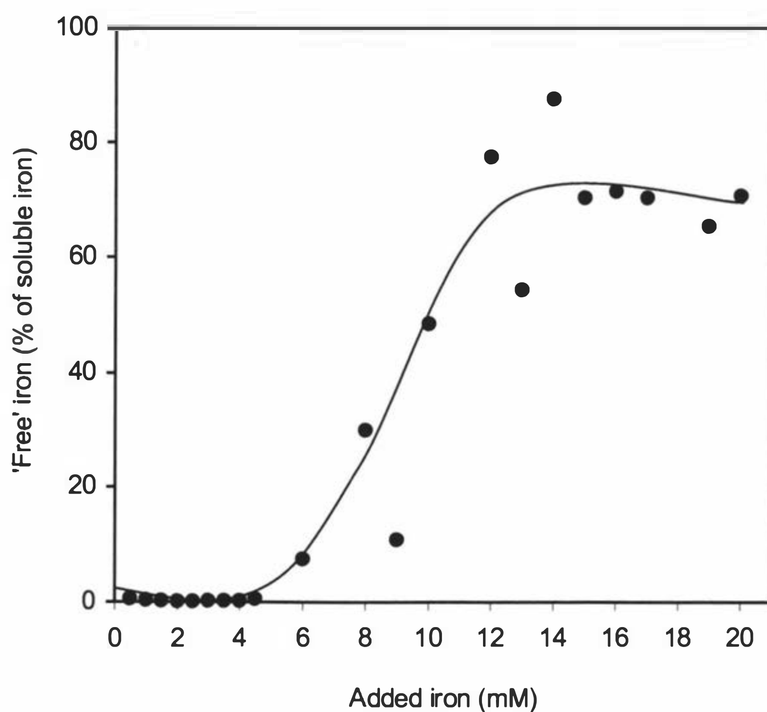


Figure 4.13. Amount of 'free' soluble iron (i.e. in the ultrafiltration permeate) after ultrafiltration of ferrous sulphate–sodium caseinate mixtures (dissolved in 50 mM HEPES buffer at pH 6.6).

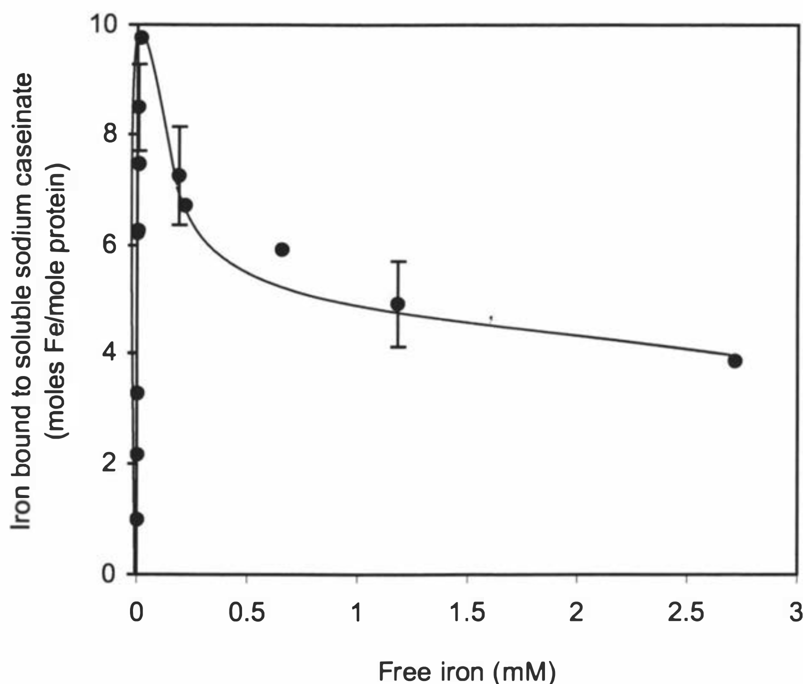


Figure 4.14. Binding of iron by soluble sodium caseinate dispersed in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

The binding isotherm obtained in this region, in terms of moles of iron/mole of sodium caseinate (MW of sodium caseinate was 22,000 Da) is shown in Figure 4.14. The binding of iron increased dramatically with added iron up to 4 mM as there was no free iron in the permeate (i.e. all of the added iron was bound to the soluble caseins). The plot of bound versus free iron for sodium caseinate showed maximum binding around 8–10 moles of iron/mole protein (approximately 20.4–25.5 mg iron/g protein) (Figure 4.1.4). The decrease in binding at relatively high free iron concentration was due to the iron and sodium caseinate precipitating at different rates in the iron–sodium caseinate mixtures (Figure 4.5) at above added iron concentration of 4 mM. Because the added iron precipitated faster than the sodium caseinate, the amount of soluble bound iron became small, hence the observed decrease in the extent of binding (Figure 4.14).

At ≥ 4 mM added iron, the amount of iron in the ultrafiltration permeate increased almost linearly and approximately 70% of the added iron was found in the permeate at > 14 mM (Figure 4.13). This was due to the increased precipitation of sodium caseinate, in particular the α_s- and β-caseins (Figure 4.10), which are known to bind more divalent cations than does κ-casein. This precipitation will cause an increase in the amount of the 'free' iron in the permeate because there is less soluble sodium caseinate, especially the α_s- and β-caseins, available to bind the added iron.

Previous studies on the binding of iron to sodium caseinate did not determine the maximum amount of iron bound to the soluble fraction of casein. Nelson and Potter (1979) reported that, at 20 mg added iron/g protein (at 25°C), the insoluble fraction of casein bound 8.7 mg iron/g protein. However, the maximum amount of iron that could be bound to the soluble fraction was not mentioned. A study by Gaucheron *et al* (1996) characterised iron-supplemented caseins up to an iron concentration of only 1.5 mM (approximately 3.4 mg Fe/g protein) to prevent precipitation of the protein at higher added iron concentrations. However, a study on the binding of iron to an individual casein (β-casein) by Baumy and Brule (1988b) did state the maximum amount of bound iron. They reported that, at pH 6.6 and very low ionic strength, 6.6 moles of Fe^{2+} were

bound per mole of β-casein. This amount cannot be compared directly with the result obtained in the present study because of the different casein fractions used.

4.4.2. Binding of Iron to Sodium Caseinate in the Insoluble Fraction

Above a concentration of 4 mM added ferrous sulphate, analysis of the binding of iron to sodium caseinate became complex. When more iron was added, sodium caseinate started to precipitate and, as a result, some of the iron also co-precipitated with the protein on centrifugation (Figure 4.5). The amount of iron that co-precipitated with the protein (i.e. the insoluble iron), as well as the insoluble protein, increased with increasing additions of ferrous sulphate. The amount of iron bound to sodium caseinate in the insoluble fraction was not determined because the amounts of iron and caseinate in the insoluble fraction could not be quantified.

4.4.3. Binding Sites and Binding Constants

The binding data were analysed using Scatchard and Klotz plots to calculate the apparent association constant (K_{app}) and the maximum number of binding sites (n). K_{app} is a measure of average binding strength for the interactions of the various binding sites in caseinate with iron and gives an indication of the affinity of iron for the binding sites on the protein molecule.

The equation used for the Scatchard plot was:

$$\nu/\mathrm{A} = K_{app}(\mathrm{n} - \nu)$$

and the equation for the Klotz plot (or double reciprocal plot) was:

$$1/\nu = 1/\mathrm{n} + (1/\mathrm{n}K_{app})(1/\mathrm{A})$$

where ν is the moles of iron bound per mole of protein and A is the moles of free iron.

In the Scatchard plot, the K_{app} value is obtained from the slope of the linear portion (Dahlquist, 1978) and the estimated maximum number of binding sites (n) is calculated from the intercept of the plot on the x-axes (intercept = $K_{app}.\mathrm{n}$). In the Klotz plot, the

values of n and K_{app} are calculated from the slope ($1/nK_{app}$) and intercept ($1/n$) of the straight-line portion of the curve (O'Neill, 1996).

The Scatchard plot for the binding of iron to sodium caseinate is shown in Figure 4.15. This plot showed upward convexity at low molar ratios of iron to casein. Two straight lines were fitted to the plot. The first straight line (I) showed the specific binding sites of the iron on sodium caseinate, with high affinity for iron. The number of binding sites was found to be approximately 14, with a log K_{app} value of 5.3. The second straight line (II) (at high molar ratios of iron to casein) probably showed the presence of some non-specific binding sites. The nature of the Scatchard plot, which showed upward convexity at low molar ratios of iron (Nichol and Winzor, 1976), could have been due to the iron-induced association of casein because of the observed aggregation of caseins with increased addition of iron.

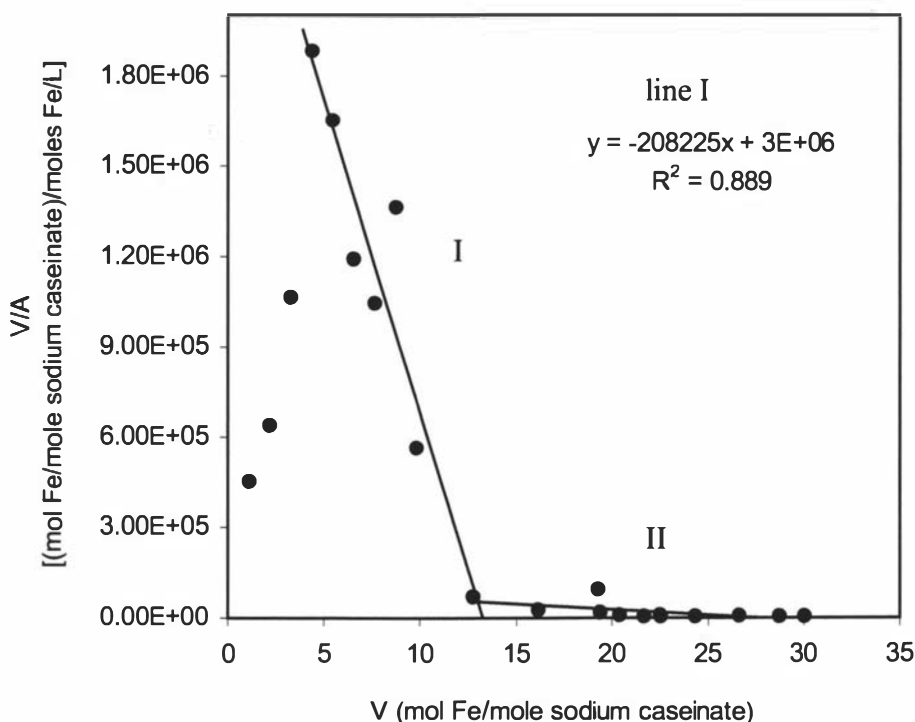


Figure 4.15. Scatchard plot for the binding of iron to sodium caseinate dispersed in 50 mM HEPES buffer at pH 6.6. The first 3 data points which showed upward convexity were excluded from the regression analysis for line I.

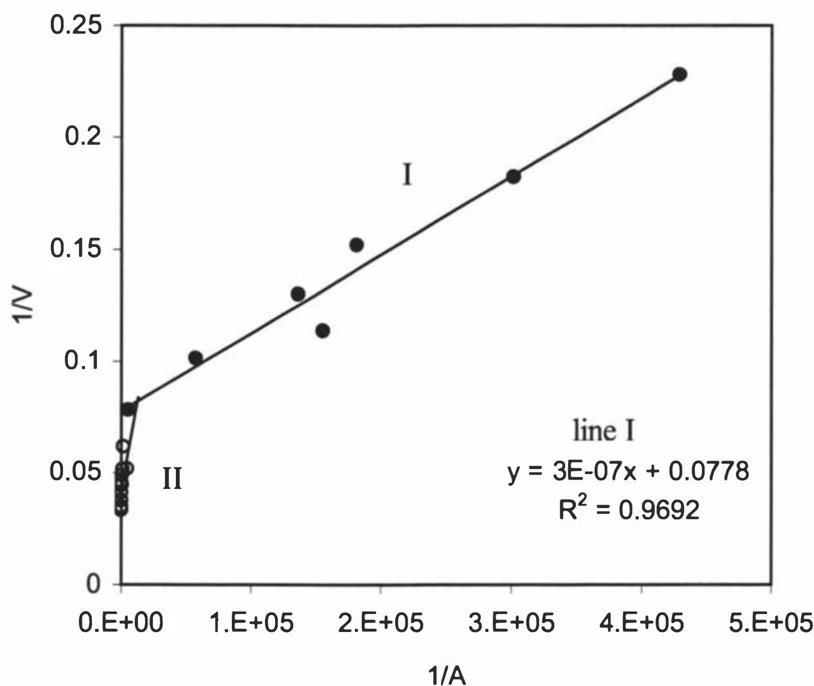


Figure 4.16. Klotz plot for the binding of iron to sodium caseinate dispersed in 50 mM HEPES buffer at pH 6.6. All data points in line I were used in the regression analysis.

The Klotz plot for the binding of iron to sodium caseinate is shown in Figure 4.16. From this plot, it was found that the number of binding sites (n) was approximately 13, with a log K_{app} value of 5.4. Therefore, there was good agreement between the two types of binding analysis.

4.4.4. Effect of pH on the Binding of Iron to Sodium Caseinate

The effect of pH on the binding of iron by sodium caseinate was investigated at an iron concentration of 2 mM. At this concentration, sodium caseinate could bind almost all the added iron without precipitation.

Changing the pH in the range from 5.5 to 7.0 did not significantly affect the solubility of sodium caseinate in the iron–sodium caseinate mixtures (Figure 4.17). However, lowering the pH from 5.5 to 3.0 caused a considerable decrease in the solubility of

sodium caseinate, from approximately 80% at pH 5.5 to less than 10% at pH 3.0. This trend partly agrees with Nelson and Potter (1979), who studied the effect of the addition of iron on the solubility of sodium caseinate (dissolved in water) when the pH was changed from 4.0 to 10.0. They found that sodium caseinate, in the absence of iron, was approximately 90% soluble in the pH range 7.0–10.0 and that the solubility decreased to approximately 70, 20 and 0% at pH 6.0, 5.0 and 4.0 respectively. The presence of iron did not affect the solubility of caseinate at pH 10.0 but it decreased the solubility of sodium caseinate even further to approximately 70 and 45% at pH 8.0 and 7.0 respectively; at < pH 6.0, the solubility of sodium caseinate in the presence of iron was extremely low.

The solubility of the iron added to sodium caseinate remained largely unaffected in the pH range 5.5–7.0 (Figure 4.17). Decreasing the pH from 5.5 to 5.0 markedly decreased the solubility of iron, but a further decrease in pH resulted in a large increase in the solubility of iron. Only approximately 40% of the added iron was soluble at pH 5.0, but approximately 80% of the added iron was soluble at pH 3.0–3.5. Hence, it is possible that, at pH 5.0, the added iron was bound to the sodium caseinate, and then co-precipitated with the caseinate. In contrast, the large amount of soluble iron at pH 3.0 and pH 3.5 suggests that the sodium caseinate was unable to bind added iron at these pH values.

Previous studies have shown that caseinate is soluble at pH values < 3.5 (Mulvihill and Fox, 1989; Mulvihill, 1992) and Vojdani (1996) reported that the solubility of casein (0.1% w/v in water) is usually > 80% at pH 2.0 and 3.0. However, under the conditions used in this present work (50 mM HEPES buffer with 0.1 M NaCl present), sodium caseinate was not soluble at these pH values.

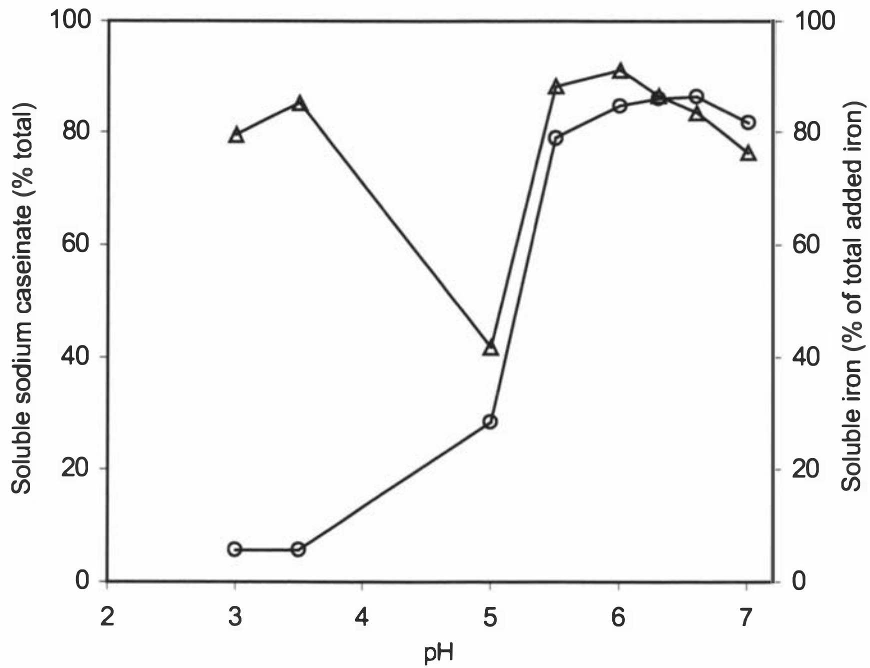


Figure 4.17. Effect of pH on the solubility of 1% sodium caseinate solution (o) and iron (△) in iron–sodium caseinate mixtures after addition of 2 mM ferrous sulphate (in 50 mM HEPES buffer).

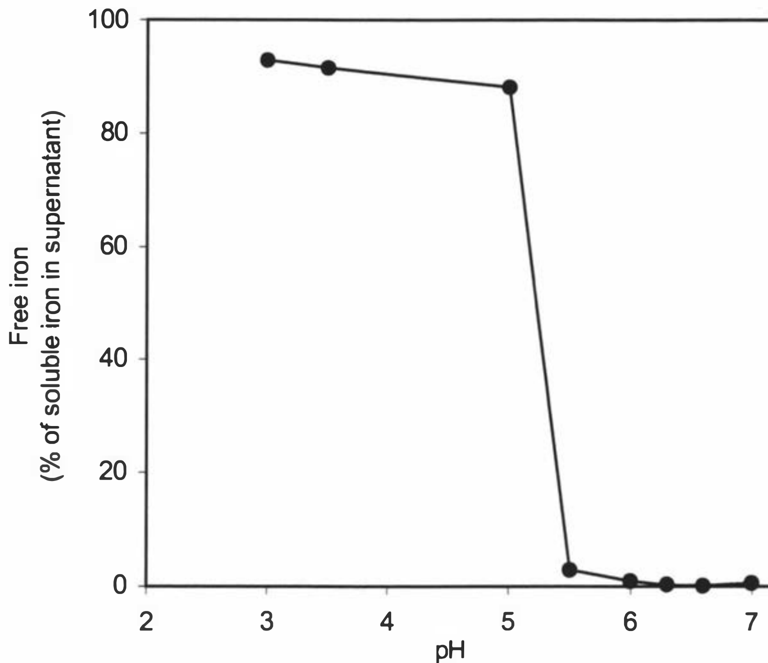


Figure 4.18. Effect of pH on the free iron (iron in the ultrafiltration permeate) in iron–sodium caseinate mixtures (in 50 mM HEPES buffer) (calculated as a percentage of the soluble iron in the supernatant). The total concentration of iron in the original solution was 2 mM.

In view of this apparent disagreement, the solubility of sodium caseinate in the pH range from 2.5 to 5.5 was investigated further. The 1% sodium caseinate was dissolved in water and in 50 mM HEPES buffer in the presence and in the absence of 0.1 M NaCl. The solubility of 1% sodium caseinate, dissolved in water, at pH 2.5 and pH 3.0, was approximately 80% (Figure 4.19). This suggested that the insolubility of sodium caseinate in HEPES buffer at low pH was probably due to the presence of NaCl in the buffer. When sodium caseinate was dissolved in 50 mM HEPES buffer in the absence of NaCl, the solubility increased markedly at pH 2.5 and 3.0 (Figure 4.19). This confirmed that the presence of 0.1 M NaCl in the HEPES buffer was responsible for the low solubility of sodium caseinate at low pH values. Addition of NaCl could lead to protein aggregation because the ions shield the repulsive forces that hold the proteins apart in solution. Furthermore, salt ions compete with the charged proteins for water molecules, reducing hydration cover and, consequently, increasing protein–protein interactions (Silva *et al*, 2003).

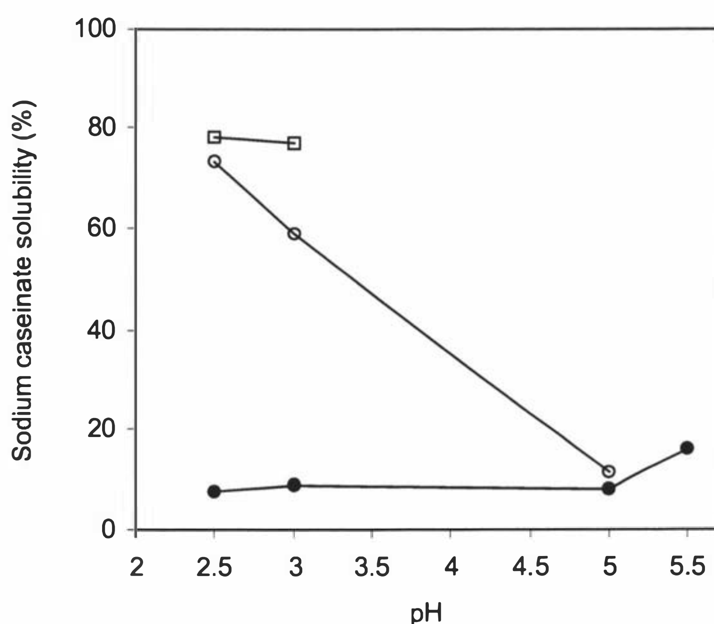


Figure 4.19. Solubility of 1% sodium caseinate in water (□) in the absence of 2 mM ferrous sulphate; solubility of 1% sodium caseinate in 50 mM HEPES buffer in the presence (•) and in the absence (o) of 0.1 M NaCl, after the addition of 2 mM ferrous sulphate.

As expected, lowering the pH caused an increase in the amount of soluble free iron in the ultrafiltration permeate (Figure 4.18). In the pH range from 5.5 to 7.0, there was virtually no free iron. At < pH 5.0, the amount of free iron increased markedly to almost 90%. As a result, the amount of iron bound to sodium caseinate also changed when the pH was changed (Figure 4.20). Approximately 12 mg Fe was bound per g of protein at pH between 5.5 and 7.0. However, this value decreased to approximately 4–5 mg Fe/g protein at pH < 5.5. It must be noted that, because the concentrations of soluble sodium caseinate in these pH regions were low, the calculation may have been inaccurate.

The amount of iron bound to caseinate did not seem to be affected by a change in pH from 5.0 to 7.0. This finding agrees with the results of Baumy and Brule (1988b), who investigated the effect of pH on the binding of iron to β-casein in the pH range 5.0–8.0, and found that the amount of iron bound to protein was independent of pH in this pH range.

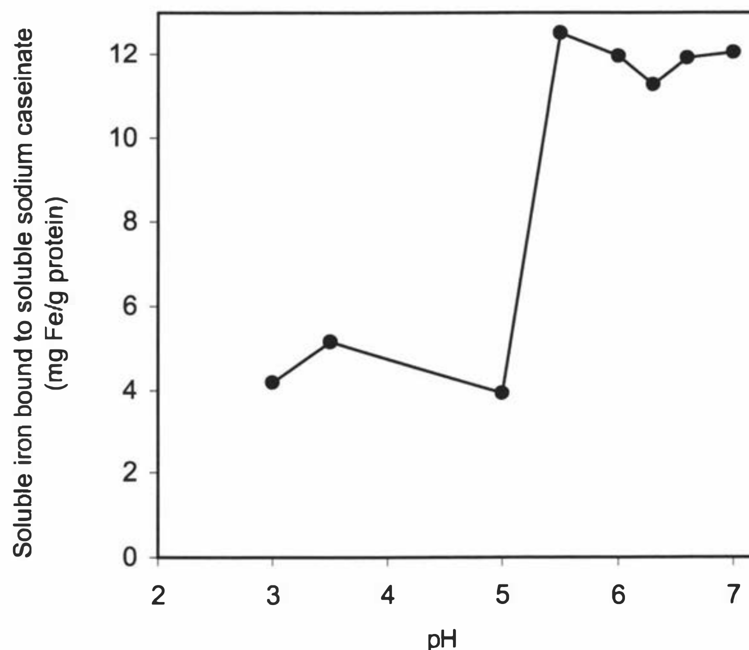


Figure 4.20. Effect of pH on the amount of iron bound to soluble sodium caseinate, in 50 mM HEPES buffer.

There are conflicting results in the literature on the effect of pH on the binding of iron to sodium caseinate. A study by Gaucheron *et al* (1996) showed that the bound iron (the iron concentration used was 1.5 mM or 3.4 mg Fe/g protein) was unmodified by a pH decrease between 3.7 and 6.5 because no iron was found in the ultrafiltration permeates. In contrast, Nelson and Potter (1979) found that the binding of iron (at 20 mg Fe/g protein) by caseinate changed with a change in pH. The amount of iron bound to caseinate was found to be maximum at pH 8.0 and almost no iron was bound to caseinate at pH $\leq$ 6.0.

The iron associated with sodium caseinate was thought to be bound to the casein by co-ordination bonds and not by ionic bonds because the link between iron and caseins is stable to changes in pH (from 3.7 to 6.5) (Gaucheron *et al*, 1996). Baumy and Brule (1988b) also stated that Fe^{2+} is probably bound to β-casein through ionic links with phosphoserine and also co-ordination links with NH_2, CO_2H, CONH and H_2O.

4.4.5. Effect of Ionic Strength on the Binding of Iron to Sodium Caseinate

Changes in ionic strength from 0.1 to 0.5 M by the addition of NaCl to the iron–sodium caseinate mixture (50 mM HEPES buffer – pH 6.6, 2 mM added iron) caused a slight decrease in the solubility of sodium caseinate (Table 4.2). The amount of 'free' iron found in the ultrafiltration permeate was only affected slightly when the ionic strength was increased from 0.25 to 0.5 M. This was probably due to the slight decrease in the solubility of sodium caseinate at 0.5 M ionic strength, which caused more release of iron into the permeate. As a result, the amount of soluble iron bound to soluble sodium caseinate was not significantly affected by an increase in ionic strength.

Gaucheron *et al* (1997a) investigated the effect of ionic strength on iron (Fe^{3+}) bound to sodium caseinate. They reported that casein molecules bound Fe^{3+} strongly because an increase in NaCl concentration, in the range from 0 to 0.12 M, to casein solutions containing 1.5 mM iron did not affect the amount of bound Fe^{3+}. This was due to the strong affinity of casein for iron. Baumy and Brule (1988b) found that the binding

ability of Fe^{2+} to an individual casein (β-casein) was not altered by ionic strength (from 0 to 0.1 M) because of the presence of co-ordination links (Section 4.4.4). The result obtained in the present study could not be compared directly with the results from Baumy and Brule (1988b) or Gaucheron *et al* (1997a) because of the different source of casein used and the different range of ionic strength. However, the result in the present study also showed that the amount of iron bound to sodium caseinate was not affected by a change in ionic strength.

Table 4.2. Effect of the ionic strength of iron–sodium caseinate mixtures (containing 2 mM ferrous sulphate, in 50 mM HEPES buffer at pH 6.6) on the ability of caseinate to bind iron

Ionic strength (M)	Soluble sodium caseinate (% of total)	'Free' iron (% of soluble iron)	Soluble iron bound to soluble sodium caseinate (mg Fe/g protein)
0.1	83.6	0.2	13.0
0.25	80.9	0.2	13.2
0.5	73.1	0.4	13.3

4.5. Solubility of Iron and WPI in Iron–WPI Mixtures

The solubility of iron in the absence and presence of WPI is shown in Figure 4.21. In the presence of WPI, the solubility of iron was considerably greater at low ferrous sulphate concentrations, but decreased gradually with increasing levels of ferrous sulphate in the mixture. The solubility of iron reached a constant level of approximately 60% in the presence of WPI at > 10 mM added iron. In contrast, the solubility of iron in the absence of WPI increased from low values (approximately 10%) with increasing added iron concentrations (Figure 4.21).

The solubility of WPI in iron–WPI mixtures is shown in Figure 4.22. Although there were some variations in the solubility of WPI with added iron concentrations, the

solubility varied between only 80 and 90%. Therefore, the addition of iron did not cause significant precipitation of WPI.

The turbidity of iron–WPI mixtures showed an increase with increasing added iron concentration at pH 6.6 (Figure 4.23). However, the turbidity values were low (less than 0.6) compared with the turbidity values of iron–sodium caseinate mixtures (as high as 2.5) (Figure 4.6). This indicates that either the extent of aggregation was lower or the sizes of the aggregates were smaller compared with those in sodium caseinate.

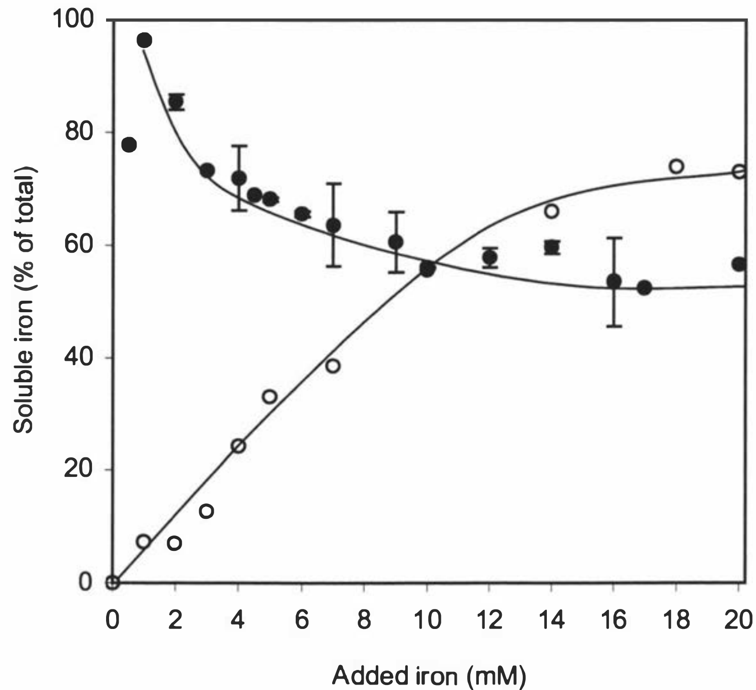


Figure 4.21. Solubility of iron in the absence (o) and in the presence (•) of WPI dispersed in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

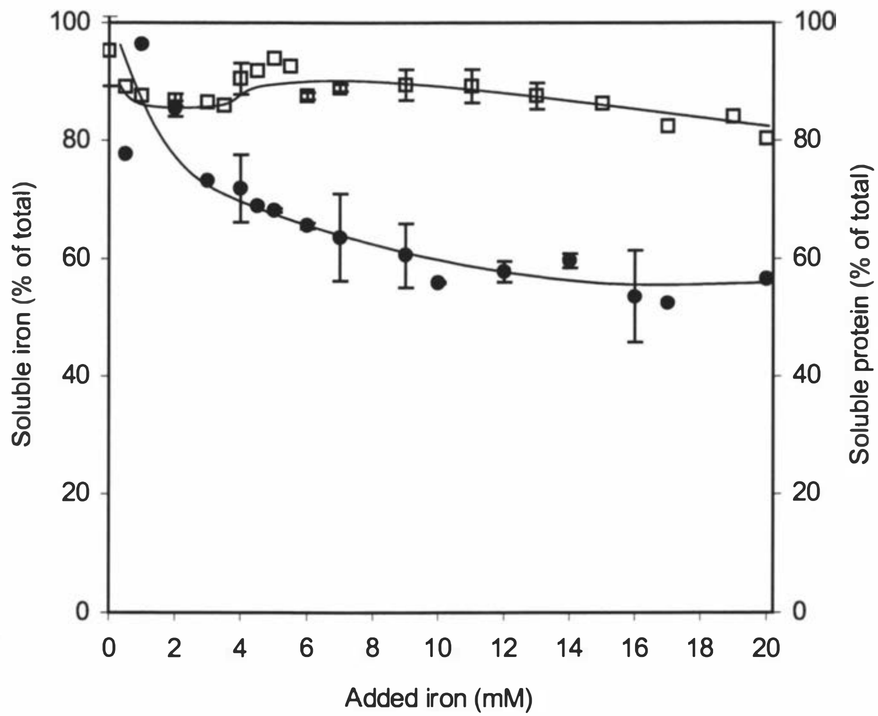


Figure 4.22. Solubility of iron (•) and WPI (□) in iron–WPI mixtures dispersed in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

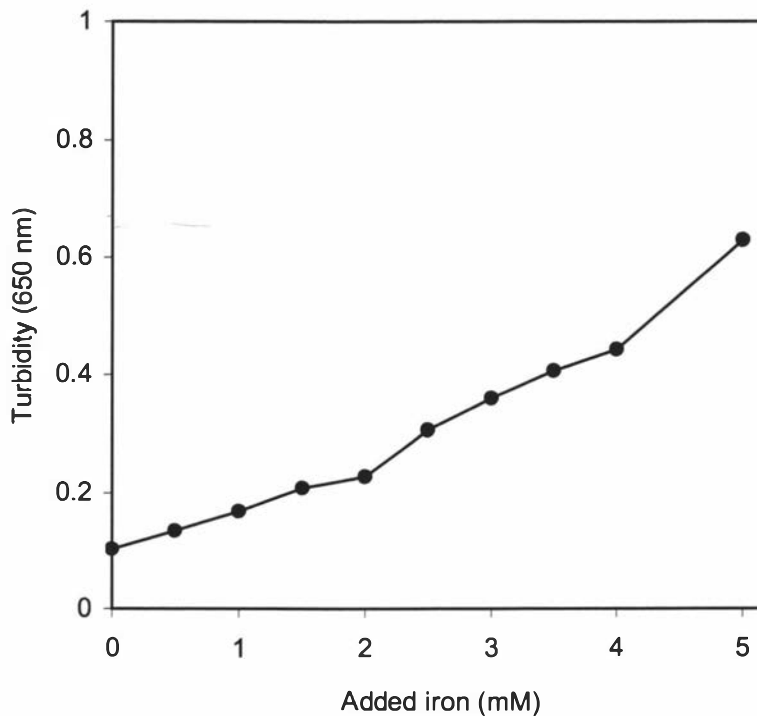


Figure 4.23. Turbidity (at 650 nm) of iron–WPI mixtures as a function of added iron concentration. pH 6.6

4.5.1. Solubility of Individual Whey Proteins in Iron–WPI Mixtures

The SDS-PAGE results obtained for iron–WPI mixtures at various levels of added iron showed that the intensity of both the β-Lg and α-La bands in the mixtures was not affected by the addition of iron (Figure 4.24). This result confirms that addition of iron did not affect the solubility of WPI in iron–WPI mixtures.

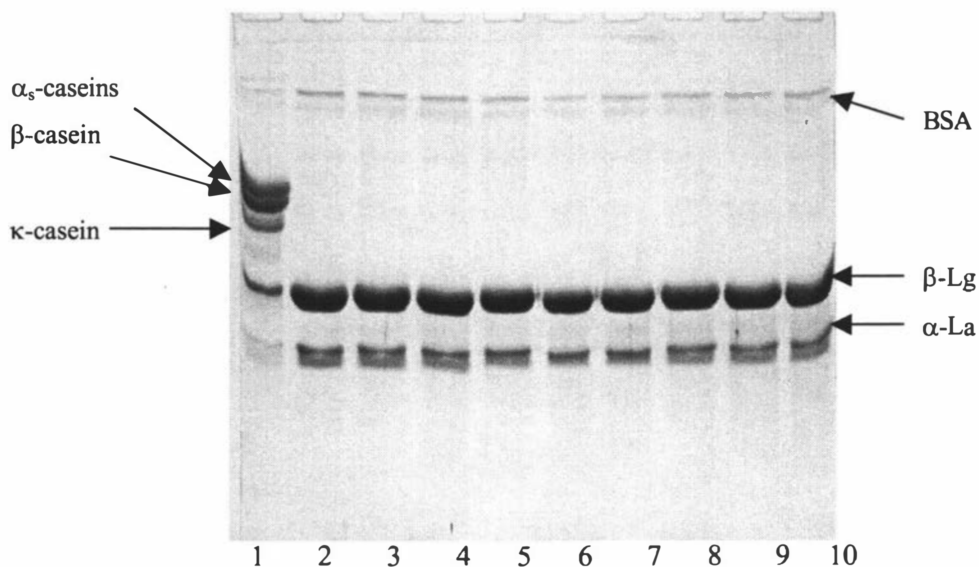


Figure 4.24. SDS-PAGE pattern of: skim milk (1), WPI solution without addition of iron before centrifugation (2), the supernatant after centrifugation (3), and supernatants obtained from iron–WPI mixtures after centrifugation with addition of iron at 2 mM (4), 4 mM (5), 6 mM (6), 8 mM (7), 10 mM (8), 16 mM (9), 20 mM (10). Samples were dissolved in 50 mM HEPES buffer at pH 6.6.

4.6. Binding of Iron to WPI

The distribution of iron between the soluble and the insoluble protein fractions in WPI was calculated as described in Section 4.4 and is shown in Figure 4.25. The iron bound to the soluble protein fraction in WPI decreased from approximately 90% to

approximately 60% at up to 3.5 mM added iron, and the proportion was constant at approximately 60% at ≥ 3.5 mM added iron. It was found that the solubility of WPI was not affected significantly by the addition of iron (Figure 4.22). Hence, most of the iron present in the insoluble fractions was not iron associated with the whey proteins but was insoluble iron on its own.

4.6.1. Binding of Iron to WPI in the Soluble Fraction

The respective proportions of free soluble iron and iron bound to soluble protein were determined using ultrafiltration (Figure 4.11). It was found that a small amount of iron (< approximately 10% of the soluble iron) was found in the permeate as free (unbound) iron up to a concentration of approximately 3.5 mM added iron (Figure 4.26). This indicates that, in this region, most of the iron was bound to the soluble whey proteins under the experimental conditions in the present study. However, as the added iron concentration increased above 3.5 mM, the amount of free soluble iron increased until it reached approximately 60% of the total soluble iron at approximately 20 mM added iron.

The binding isotherm for iron bound to WPI, in terms of moles of iron per mole of protein (MW of WPI was 18,000 Da), is plotted in Figure 4.27. The curve showed an almost linear increase in the amount of iron bound to soluble WPI in the region from 0 to 7 mM added iron, which gave approximately 7 moles bound Fe/mol protein. The binding almost levelled off above 7 mM added iron.

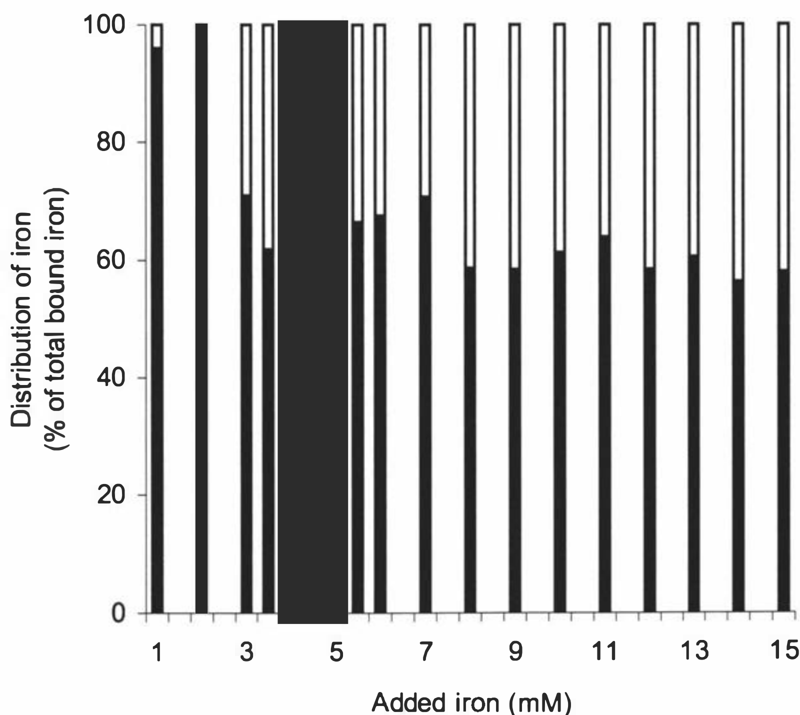


Figure 4.25. Distribution of bound iron (as a proportion of the total bound iron) between the soluble fraction (■) and the insoluble fraction (□) of WPI dissolved in 50 mM HEPES buffer at pH 6.6.

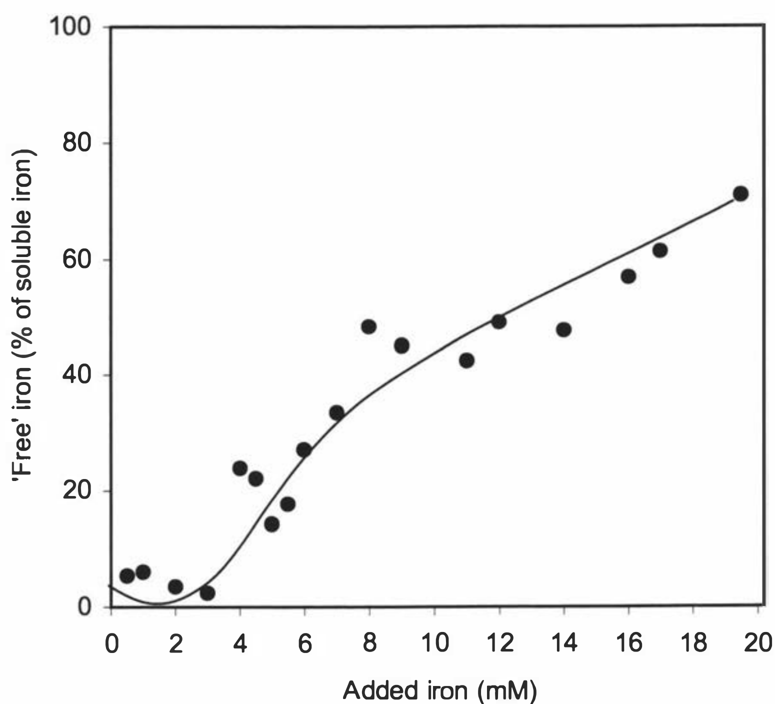


Figure 4.26. Amount of 'free' soluble iron (i.e. in the ultrafiltration permeate) after ultrafiltration of ferrous sulphate–WPI mixtures in 50 mM HEPES buffer at pH 6.6.

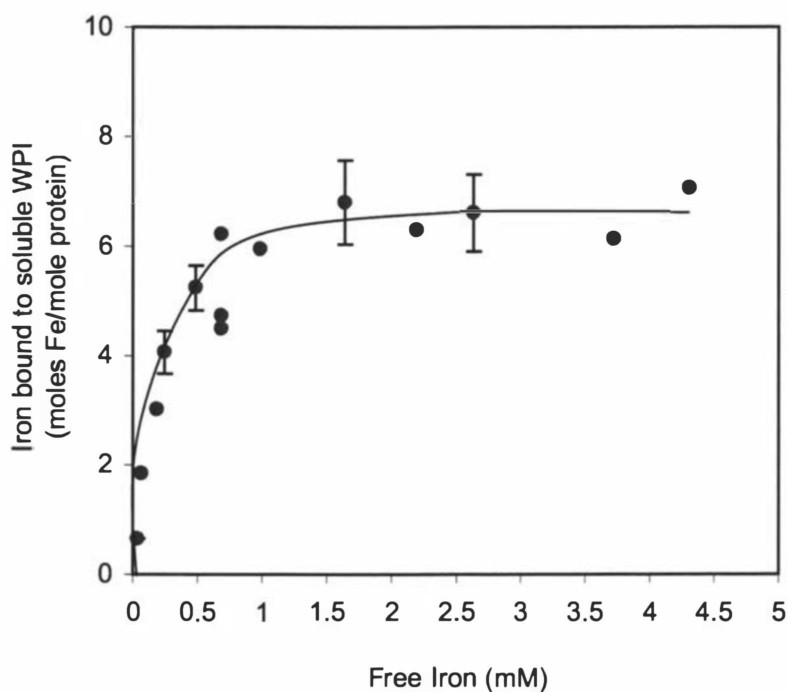


Figure 4.27. Binding of iron by soluble WPI in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

4.6.2. Binding of Iron to WPI in the Insoluble Fraction

Addition of iron to WPI solution caused only a slight decrease in the solubility of the whey proteins. In contrast, the precipitation of iron in iron–WPI mixtures was marked (Figure 4.22). Because of this, in the insoluble fraction, the iron may have become insoluble on its own and not because it was bound to the whey proteins. At low levels of added iron (1 mM), only approximately 10% of the iron was associated with the insoluble fraction of WPI (Figure 4.25). This proportion increased as the added iron increased and approximately 40% of the iron was associated with the insoluble fraction of WPI at > 3.5 mM added iron. However, this proportion may have been due to the insolubility of the iron rather than to the iron bound to the whey proteins.

4.6.3. Binding Sites and Binding Constants

The Scatchard plot (Section 4.4.3) for the binding of iron to WPI could be fitted to two straight lines with a few outliers (Figure 4.28). The first straight line showed the specific affinity sites for iron, and the second line showed some non-specific binding sites for iron. The maximum number of binding sites (n) and the K_{app} value were calculated from the first straight line curve (I) of the plot. It was found that WPI had about 8 binding sites, with a log K_{app} value of 3.6. These values were lower than the values of n (about 14) and log K_{app} (5.3) for the binding of iron to sodium caseinate (Section 4.4.3). The results demonstrate that sodium caseinate had more binding sites than WPI to bind iron and that the affinity of caseinate to bind iron was higher than that of whey proteins.

Thus far, no data on the amount of iron bound to WPI are available in the literature to compare with the result obtained in the present study. The general contention from the studies of the binding of iron to milk is that caseins, in general, have a greater ability than whey proteins to bind iron (Demott and Dincer, 1976; Carmichael *et al*, 1975; Hegenauer *et al*, 1979c). When iron is added to milk, a competition in binding probably exists, whereby the preference for binding is to the casein fractions in milk and not to the whey protein fractions. However, individual whey proteins such as α-La and β-Lg were found to have the ability to bind iron (Baumy and Brule, 1988a).

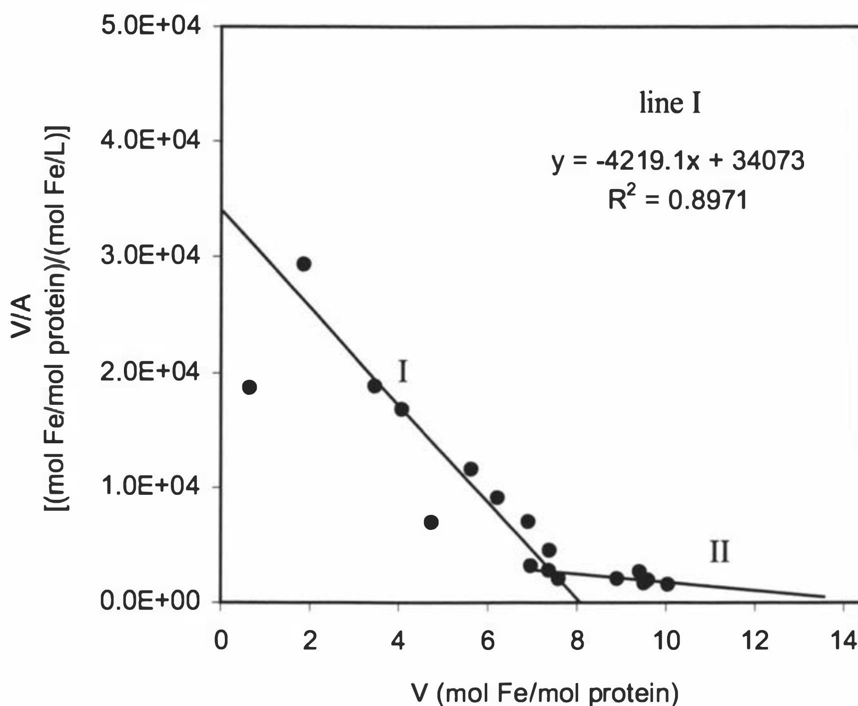


Figure 4.28. Scatchard plot for the binding of iron to WPI, in 50 mM HEPES buffer at pH 6.6. All data points in line I were used in the regression analysis.

4.6.4. Effect of pH on the Binding of Iron to WPI

The effect of pH (from 3.0 to 7.0) on the binding of iron to WPI was investigated at an added iron concentration of 2 mM, because most of the added iron was bound to the protein at this concentration.

Changing the pH of iron–WPI mixtures in the range from 7.0 to 3.0 caused a slight decrease in the solubility of the WPI from approximately 100% at pH 7.0 to approximately 90% at pH 3.0 (Figure 4.29). The solubility of iron in iron–WPI mixtures was also affected by acidification. The solubility of iron increased slightly from approximately 70% to approximately 80% from pH 7.0 to pH 5.5, and was approximately 90% at pH ≤ 5.0. Because the solubility of whey protein was affected only slightly by acidification, the increase in the solubility of iron at lower pH was due to the properties of iron, which is more soluble at lower pH (Section 4.2).

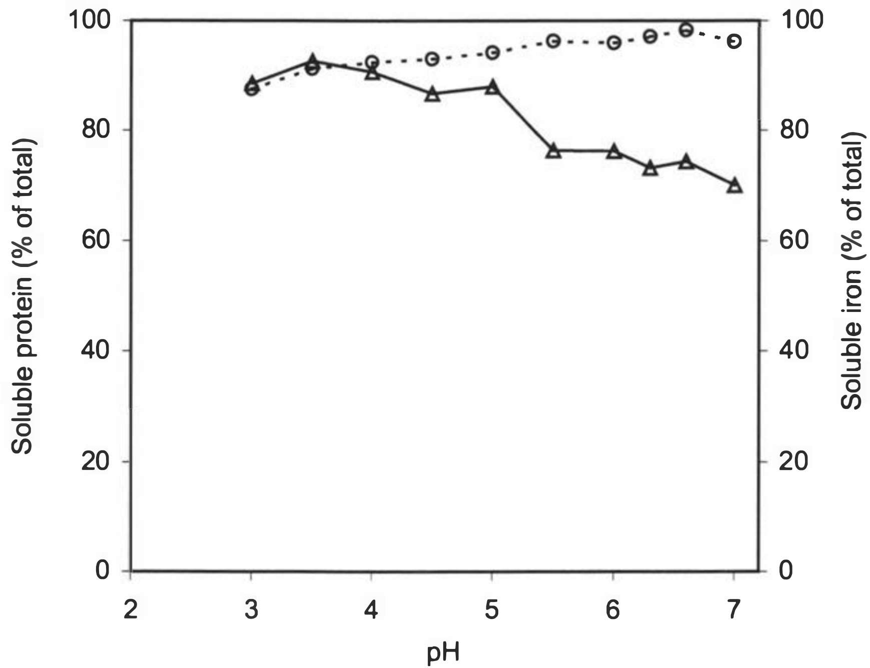


Figure 4.29. Effect of pH on the solubility of 1% WPI solution (o) and iron (△) in iron–WPI mixtures after addition of 2 mM ferrous sulphate, in 50 mM HEPES buffer.

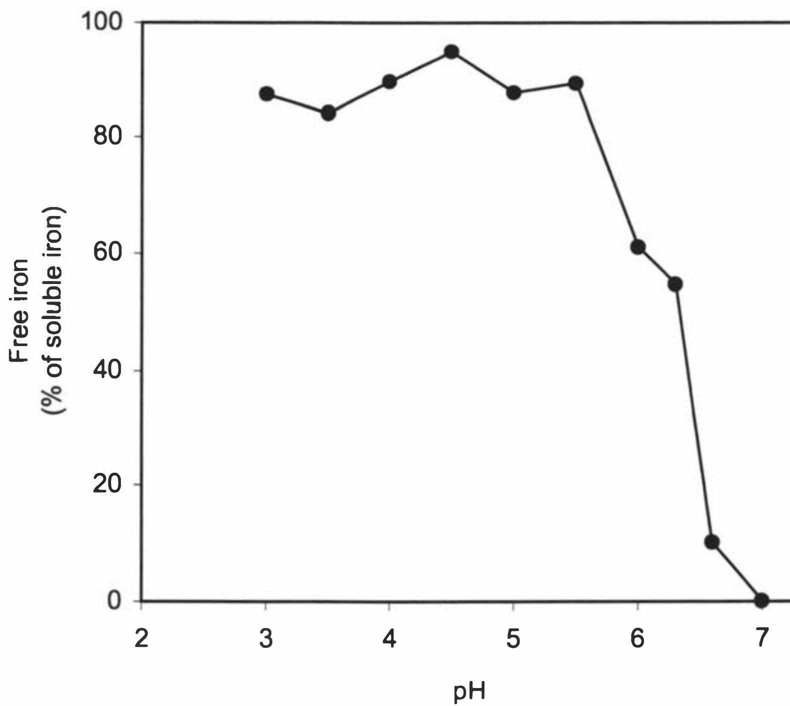


Figure 4.30. Effect of pH on the free iron (iron in the ultrafiltration permeate) in iron–WPI mixtures (in 50 mM HEPES buffer) (calculated as a percentage of the soluble iron in the supernatant). The total iron concentration in the original solution was 2 mM.

Acidification of iron–WPI mixtures also caused a marked increase in the amount of soluble free (unbound) iron in the ultrafiltration permeate (Figure 4.30). The amount of free iron was very low at pH 7.0 and increased rapidly to approximately 90% as the pH was decreased to 5.5. When the pH was decreased further to 3.0, the amount of free iron remained at approximately 90%. The change in the amount of free iron reflected the change in the amount of iron bound to WPI (Figure 4.31). There was approximately 8 mg bound Fe/g protein at pH 7.0. This amount decreased markedly to approximately 1 mg Fe/g protein as the pH decreased to 5.5 and was only approximately 1–2 mg Fe/g protein at pH ≤ 5.5.

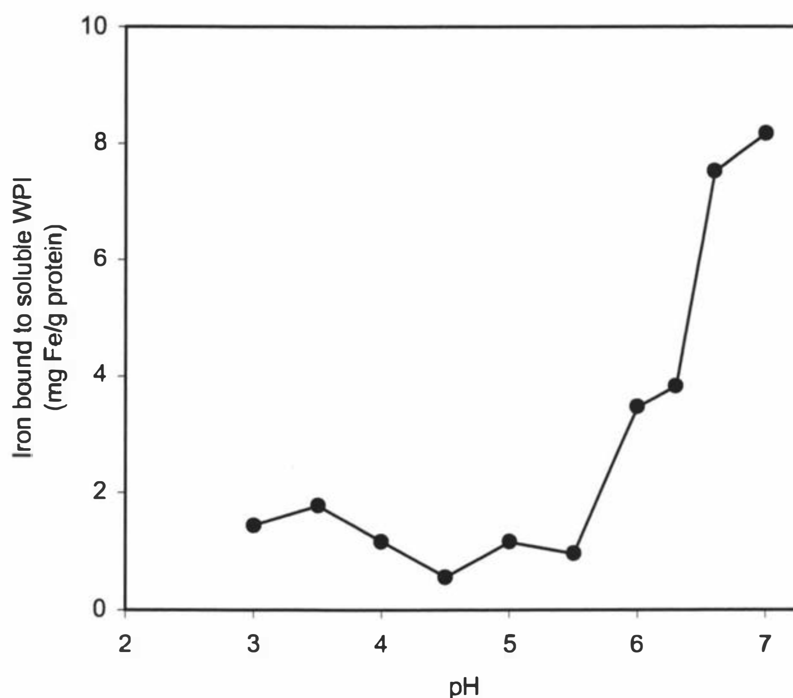


Figure 4.31. Effect of pH on the amount of iron bound to soluble WPI, in 50 mM HEPES buffer.

These results indicate that, although a decrease in pH from 7.0 to 3.0 caused only a slight change in the solubility of the whey proteins and the iron in iron–WPI mixtures, acidification caused a marked decrease in the ability of whey protein to bind iron. As

the pH was lowered to 3.0, the ability of whey protein to bind iron was markedly reduced (Figure 4.31), which caused a large increase in the amount of free soluble iron.

No published study has specifically investigated the effect of pH on the binding of iron to WPI. However, a study by Baumy and Brule (1988a) investigated the effect of pH (from 6.6 to 5.0) on the binding of iron to individual whey proteins – β-Lg and α-La. It was found that the ability of α-La and β-Lg to bind iron decreased as the pH was decreased. At pH 6.6 (ionic strength < 0.01 M), α-La and β-Lg could bind 6 ions and 3.5 ions of Fe^{2+} respectively; this ability decreased to approximately 1 and approximately 1.5 ions of Fe^{2+} at pH 5.0, for α-La and β-Lg respectively. As the WPI used in the present study consisted mostly of α-La (24.3%) and β-Lg (70.4%) (from SDS-PAGE results), it is not surprising that the binding results were in qualitative agreement with those of Baumy and Brule (1988a).

4.6.5. Effect of Ionic Strength on the Binding of Iron to WPI

The effect of ionic strength (from 0.1 to 0.5 M) on the binding of iron to WPI was also investigated at an added iron concentration of 2 mM by the addition of NaCl to iron–WPI mixtures.

The increase in ionic strength of iron–WPI mixtures caused a very slight decrease in the amount of soluble protein (Table 4.3). The amount of free iron in the permeate increased slightly with increasing ionic strength from 0.1 to 0.5 M. As a result, the amount of iron bound to WPI decreased slightly as the ionic strength increased.

Again, no published data on the effect of ionic strength on the iron-binding ability of WPI exist, although Baumy and Brule (1988a) studied the effect of ionic strength on the binding of iron by α-La and β-La. They found that increasing ionic strength from 0.01 to 0.1 M (at pH 6.6) caused a decrease in the binding ability from approximately 6 to

approximately 2 ions of Fe^{2+} for α-La and from 3.5 to approximately 1 ions of Fe^{2+} for β-Lg. This decrease was much greater than that observed in this study.

Table 4.3. Effect of ionic strength on the ability of WPI to bind iron in iron–WPI mixtures (containing 2 mM ferrous sulphate, in 50 mM HEPES buffer at pH 6.6)

Ionic strength (M)	Soluble protein (% of total)	'Free' iron (% of soluble iron)	Soluble iron bound to soluble WPI (mg Fe/g protein)
0.1	94.7	12.7	8.4
0.25	93.9	15.5	7.6
0.5	92.1	14.1	7.4

4.7. Solubility of Iron and MPC in Iron–MPC Mixtures

As described earlier (Section 4.2), the solubility of iron in the absence of protein increased gradually with an increase in iron concentration (Figure 4.32). Addition of MPC markedly improved the solubility of iron at a concentration of ≤ 8 mM added iron. However, the solubility of iron at higher added iron concentrations (> 8 mM) was less in the presence of MPC than in its absence.

The solubility of proteins in the MPC solution in the absence of iron was approximately 70% (Figure 4.33). Because the structure of MPC comprises mostly of casein micelles (Mulvihill, 1992), it is likely that, even in the absence of iron, the larger casein micelles sediment upon centrifugation. Addition of iron at up to 8 mM caused a gradual decrease in the protein solubility to approximately 40%. The solubility levelled off at approximately 40% at iron concentrations ≥ 8 mM (Figure 4.33).

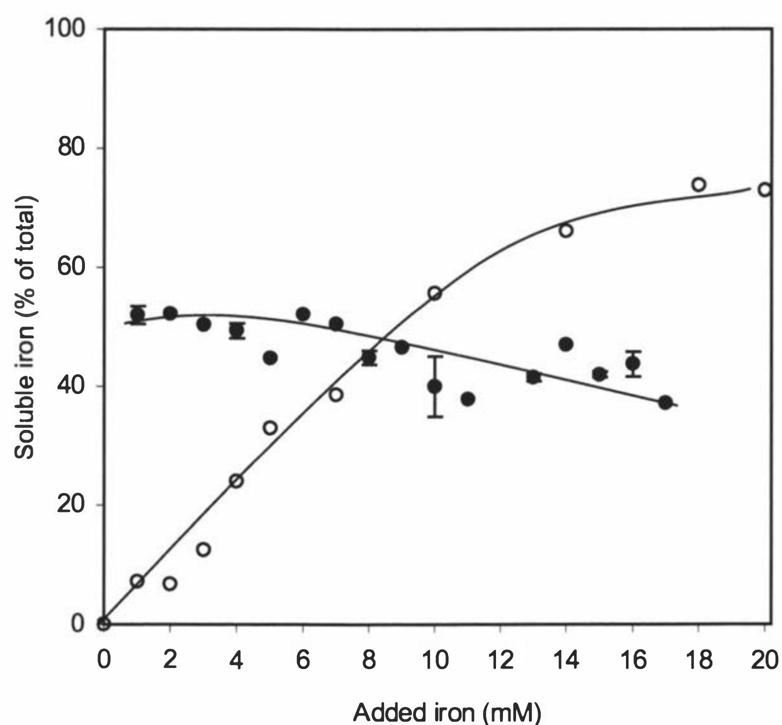


Figure 4.32. Solubility of iron in the absence (o) and in the presence (•) of MPC dispersed in 50 mM HEPES buffer, pH 6.6. Bars indicate standard errors.

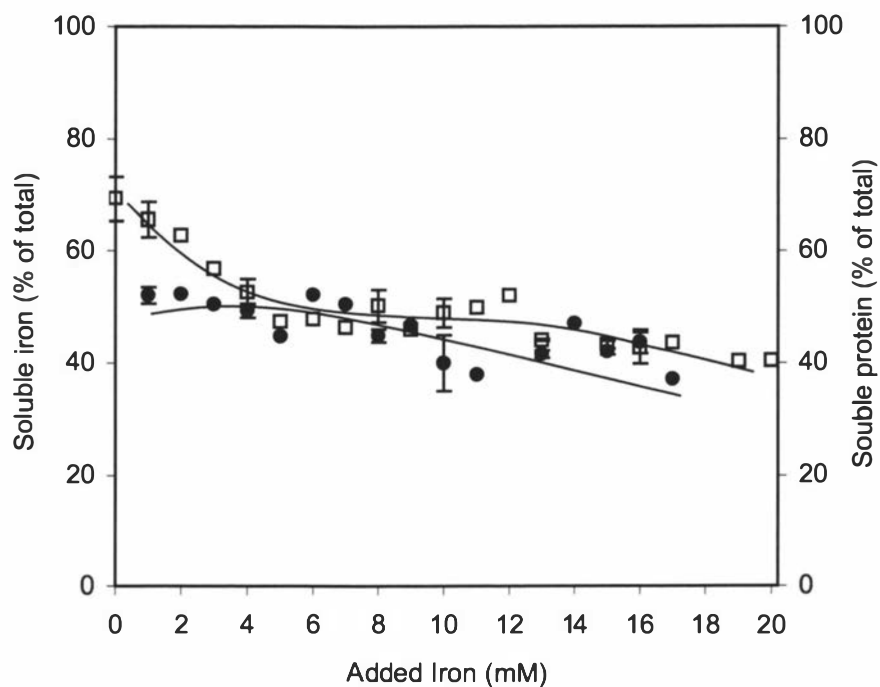


Figure 4.33. Solubility of iron (•) and MPC (□) in iron–MPC mixtures dispersed in 50 mM HEPES buffer, pH 6.6. Bars indicate standard errors.

The turbidity results presented in Figure 4.34 show that the turbidity (650 nm) increased gradually with increasing addition of iron. In other words, protein aggregation increased as the iron concentration increased. This shows that the decrease in protein solubility was probably due to the increased aggregation of casein micelles caused by protein–iron interactions.

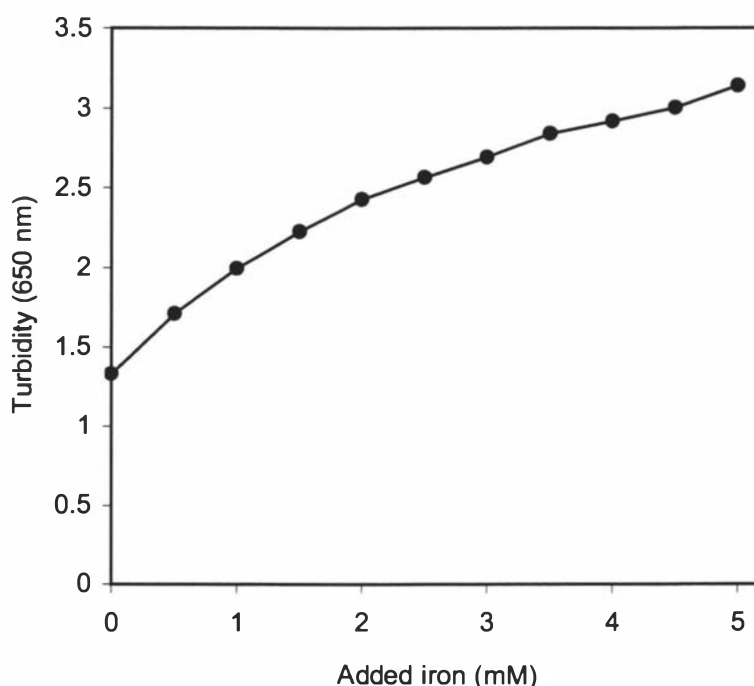


Figure 4.34. Turbidity (at 650 nm) of iron–MPC mixtures as a function of added iron concentration.

The solubility of iron at up to 8 mM added iron was also improved in the presence of MPC (Figure 4.32), indicating that the added iron associated with the soluble protein fractions in the MPC. Above an added iron concentration of 8 mM, the solubilities of MPC and iron in iron–MPC mixtures were similar at approximately 40%. This can be explained in terms of the effect of iron on the solubility of the individual proteins in MPC (Section 4.7.1).

There are no published data on the effect of the addition of iron on the solubility of MPC. Gaucheron *et al* (1997b) reported that protein precipitation occurred when iron at a concentration of 8 mM was added to skim milk (500 mL). In the present study, the solubility of MPC decreased with increased addition of iron, up to 8 mM (Figure 4.33). It is likely that the added iron interacts with the proteins in MPC, possibly with the larger casein micelles, which contain more α_s-caseins (Hekmat and McMahon, 1998). This interaction leads to either the formation of aggregates or an increase in the size of the casein micelles, which subsequently sediment under the centrifugation conditions, hence causing a decrease in the solubility of the proteins.

The binding of iron to casein micelles, when iron is added to milk samples, has been reported previously. The amount of iron associated with the casein micelles was determined by separation of the micelles by ultracentrifugation. Carmichael *et al* (1975) showed that sedimentation of non-fat milk using ultracentrifugation (42,000 *g*, 2 h) resulted in approximately 75% of the iron (added as iron(III)-nitrilotriacetate (ferric-NTA) chelate) binding to the casein micelles. Hegenauer *et al* (1979c), using the same ultracentrifugation speed, found that the casein fraction bound approximately 90% of the iron added to skim milk as ferric-NTA complex at < 1 mM added iron. Gaucheron *et al* (1997b) also found that > 89% of the iron was bound to the colloidal phase when iron (in the range 0–1.5 mM) was added to milk (the ultracentrifugation speed used was 77,000 *g*, 2 h). Similar results were obtained by Hekmat and McMahon (1998), who found that 83% of added iron sedimented and associated with the micellar milk proteins after ultracentrifugation at 55,000 *g* for 1 h.

4.7.1. Solubility of Individual Proteins of MPC in Iron–MPC Mixtures

MPC solutions containing various concentrations of added iron were centrifuged and the supernatants were analysed using SDS-PAGE.

The SDS-PAGE results showed a decrease in the intensities of the α_s-, β- and κ-casein bands with increasing added iron concentration, whereas the intensities of the β-Lg and

α-La bands were not significantly affected (Figure 4.35). Of the casein fractions, the α_s-caseins were most affected by the addition of iron (> 1 mM added iron) and became insoluble, followed by β-casein (Figure 4.36). The relative proportions of the α_s- and β-caseins continued to decrease with iron addition up to approximately 8 mM and then remained almost constant. The proportion of κ-casein was not greatly affected by the addition of iron. The relative proportions of β-Lg and α-La remained essentially constant with increasing addition of iron from 0 to 20 mM.

The solubility of MPC levelled off at approximately 40% at ≥ 8 mM added iron (Figure 4.33). This could have been due to the fact that > approximately 60% and approximately 45% of the α_s- and β-caseins respectively were insoluble above this concentration (Figure 4.36). Only κ-casein and most of the β-Lg and α-La were still soluble, which contributed to the total amount of protein measured in the supernatant. It has been reported that added iron ($FeCl_2$ or $FeCl_3$) is bound mainly to α_{s1}-casein (Gaucheron *et al*, 1997b). Reddy and Mahoney (1991b) found a conformational change in bovine α_{s1}-casein on interaction with Fe^{3+}. In addition, Vaughan and Knauff (1961) showed that the relative affinities of individual milk proteins for Fe^{3+} were α_{s1}-casein > β-casein > BSA > κ-casein > β-Lg > α-La. Therefore, because the interaction of iron with the casein fractions in MPC (especially α_s- and β-caseins) is more preferred than its interaction with the whey protein fractions, more of the added iron would co-precipitate with the caseins and hence the solubility of the iron at ≥ 8 mM added iron was only approximately 40% (Figure 4.33).

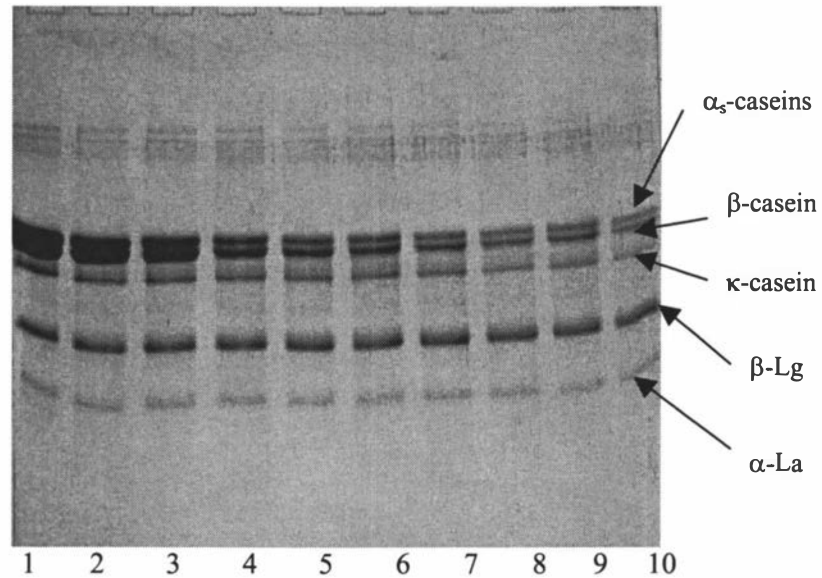


Figure 4.35. SDS-PAGE patterns of: MPC solution without addition of iron before centrifugation (1), the supernatant after centrifugation (2), and the supernatants obtained from iron–MPC mixtures with the addition of iron at 1 mM (3), 4 mM (4), 6 mM (5), 7 mM (6), 8 mM (7), 10 mM (8), 15 mM (9) and 20 mM (10). Samples were dissolved in 50 mM HEPES buffer at pH 6.6.

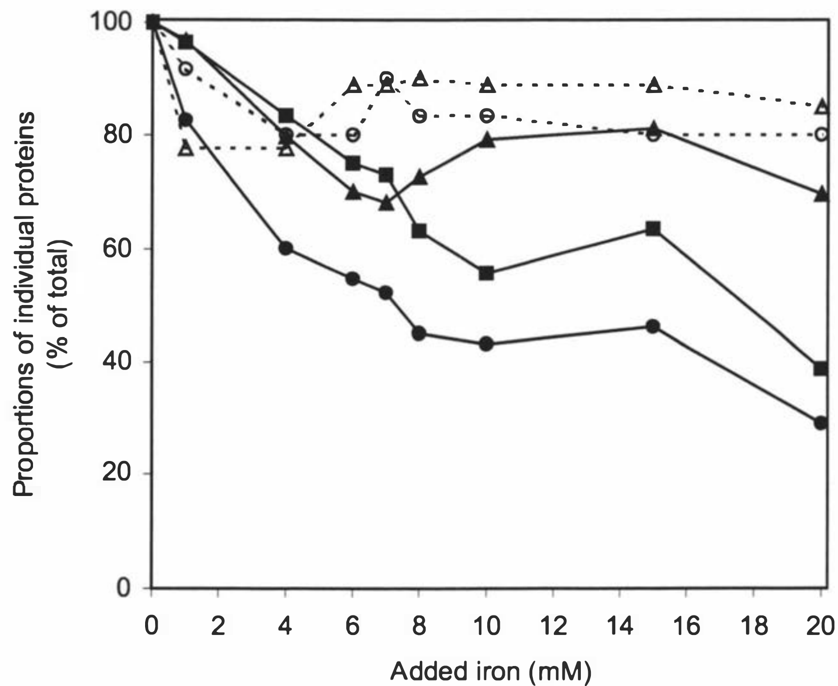


Figure 4.36. Proportions of individual proteins in the supernatant of MPC solutions: α_s- (α_{s1}- + α_{s2}-) casein (•), β-casein (■), κ-casein (▲), β-Lg (o) and α-La (△) as affected by the addition of ferrous sulphate, in 50 mM HEPES buffer at pH 6.6.

4.8. Binding of Iron to MPC

The distribution of bound iron between the soluble and insoluble fractions of the proteins in MPC is shown in Figure 4.37. Approximately 50% of the iron was bound to the soluble protein fractions of MPC at up to 8 mM added iron. As the amount of iron increased from 8 to 20 mM, the iron bound to the soluble fractions of MPC gradually decreased to approximately 20%. The relatively low proportion of iron bound to the soluble fraction of MPC was probably due to the precipitation and sedimentation of the proteins, which occurred after the addition of iron.

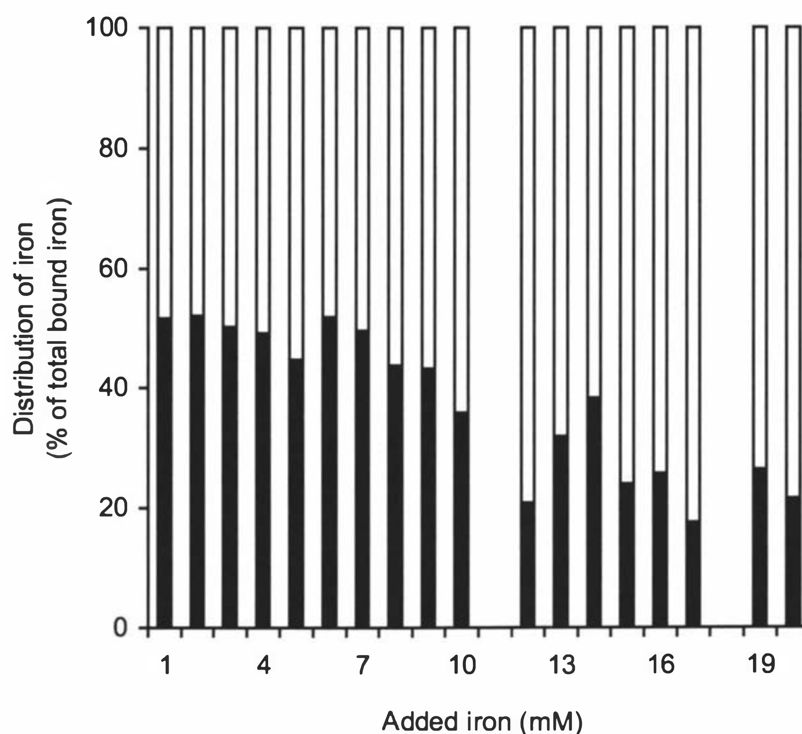


Figure 4.37. Distribution of bound iron (as a proportion of the total bound iron) between the soluble fraction (■) and the insoluble fraction (□) of MPC dissolved in 50 mM HEPES buffer at pH 6.6.

4.8.1. Binding of Iron to the Proteins in MPC in the Soluble Fraction

The respective proportions of free soluble iron and iron bound to soluble protein were determined using ultrafiltration (Figure 4.11). It was found that there was very little

free (unbound) iron in the ultrafiltration permeate at up to approximately 8 mM added iron (Figure 4.38). This indicates that, at up to this concentration, all of the added iron was tightly bound to the soluble protein part of MPC. At ≥ 8 mM added iron, the amount of free iron increased with increasing added iron until it reached approximately 70% of the total soluble iron at 20 mM added iron (Figure 4.38). The increased amount of free iron was due to the fact that the α_s- and β-caseins were only partly soluble at ≥ 8 mM added iron (Figure 4.36).

The amount of iron bound to the soluble proteins in MPC was quantified and is shown in Figure 4.39. It was found that the amount of bound iron increased with increasing added iron up to ≥ 8 mM. The binding levelled off at approximately 45 mg Fe/g protein at iron concentrations above 8 mM. No published results on the binding of iron to MPC that could be used as a comparison were available.

MPC was found to bind greater amounts of iron (approximately 45 mg Fe/g protein) than sodium caseinate (approximately 20.4 mg Fe/g protein) (Figure 4.14) despite the fact that the casein composition in MPC is lower than that in caseinate. This could have been due to the structure of the caseins in MPC, which is similar to that found in milk (Mulvihill, 1992), i.e. composed of micelles and colloidal calcium phosphate. Therefore, it is likely that MPC could bind the greatest amount of iron because of the presence of the casein micelles, and the whey protein fractions also have the ability to bind iron. A study by Gaucheron *et al* (1997b) on the addition of $FeCl_2$ and $FeCl_3$ to skim milk found that more than 89% of the iron was bound to the colloidal phase (casein micelles) with a small amount of the iron found in the aqueous phase. This phase contains whey proteins (α-La, β-Lg and lactoferrin), which also have the ability to bind iron (Baumy and Brule, 1988a).

A Scatchard plot of iron binding to MPC was not attempted because the molecular weight of MPC is not known. Hence, the number of binding sites and the binding affinity of MPC for iron could not be calculated.

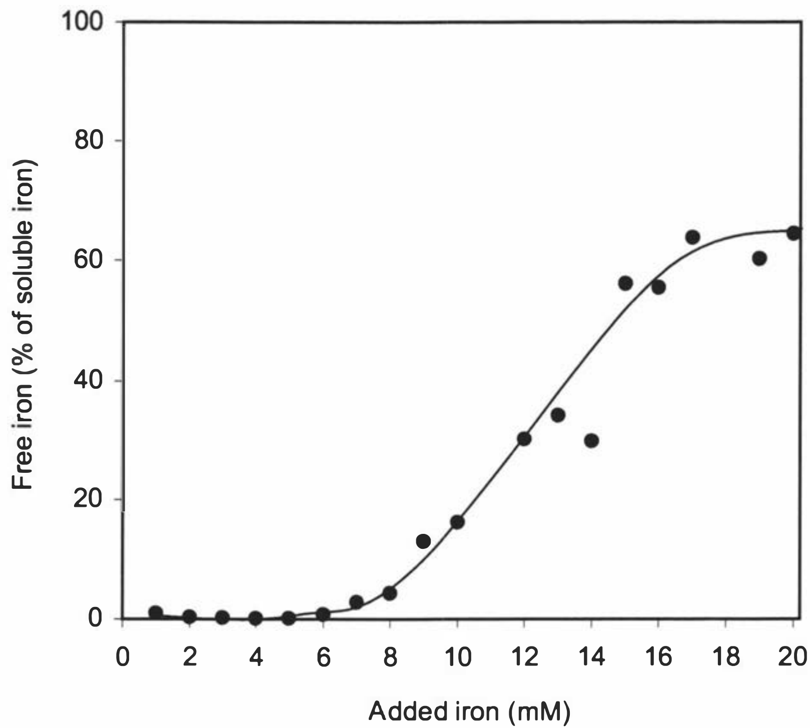


Figure 4.38. Amount of 'free' soluble iron (i.e. in the ultrafiltration permeate) after ultrafiltration of ferrous sulphate–MPC mixtures, in 50 mM HEPES buffer at pH 6.6.

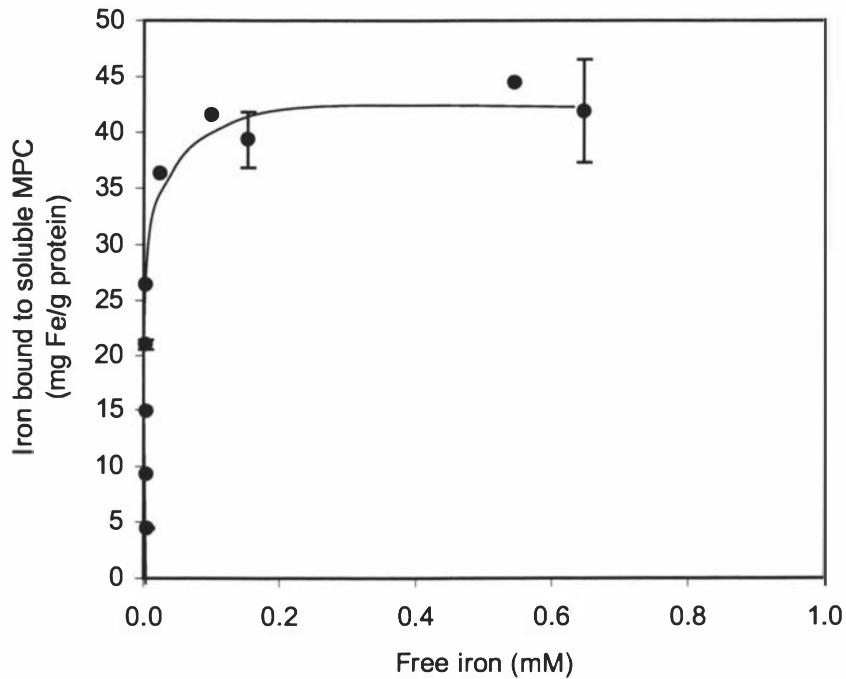


Figure 4.39. Binding of iron by soluble MPC in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

4.8.2. Binding of Iron to the Proteins in MPC in the Insoluble Fraction

When iron was added to MPC at up to a concentration of 8 mM, only approximately 50% of the added iron remained soluble and the remaining iron was insoluble upon centrifugation (Figure 4.33). This insoluble iron was likely to associate with the insoluble fraction of MPC (Figure 4.37), which probably contained the casein micelles. It is postulated that this proportion of iron was bound to the casein micelles, which co-sedimented upon centrifugation. As mentioned in Section 4.7, casein micelles have the ability to bind significant amounts of added iron (Carmichael *et al*, 1975; Hegenauer *et al*, 1979c; Gaucheron *et al*, 1997b; Hekmat and McMahon, 1998).

4.8.3. Effect of pH on the Binding of Iron to MPC

Changes in the pH from 3.0 to 7.0 of iron–MPC mixtures (at 2 mM added iron) caused changes in the solubilities of the iron and the proteins in the MPC (Figure 4.40). The solubility of the proteins in the iron–MPC mixture decreased slightly from approximately 70% to 60% as the pH was decreased from 7.0 to 6.2. The solubility then increased to approximately 80% when the pH was changed to 5.5. This was followed by a marked decrease in the protein solubility to approximately 10% as the pH was lowered to 3.0. The decrease in the solubility of MPC at pH 3.0 and 3.5 may have been due to the presence of NaCl in the buffer. When MPC was dissolved in water at pH 3.5 and 3.0, its solubility in the absence of iron was approximately 90%. However, when MPC was dissolved in HEPES buffer (containing 0.1 M NaCl) with 2 mM added iron, the solubility was only approximately 10%. Furthermore, MPC could not be dissolved in HEPES buffer without the presence of 0.1 M NaCl. Hence, the decrease in protein solubility under the conditions in the present study could have been due to the effect of NaCl on the solubility of MPC, especially at low pH.

The solubility of iron was also affected by acidification of the iron–MPC mixtures (Figure 4.40). The solubility of iron was 60% at pH 7.0 and increased to approximately 90% at pH 5.5. The solubility was approximately 60% at pH 5.0 and pH 3.5, and was

approximately 80% at pH 3.0. The amount of free soluble iron in the permeate was almost 0% when the pH was between 5.5 and 7.0 (Figure 4.41). There was a marked increase in the amount of free iron at below pH 5.5 and approximately 90% of the iron was found in the permeate at pH 3.0 and 3.5.

When the amount of iron bound to the soluble proteins in MPC was calculated, it was found that the amount increased from approximately 9 to 12 mg Fe/g protein as the pH was decreased from 7.0 to 5.5 (Figure 4.42). This slight increase may have been due to the variations in the solubilities of the iron and the proteins (for a pH range from 5.5 to 7.0) in the iron–MPC mixtures. This amount then decreased to approximately 4 mg Fe/g protein with a decrease in the pH to 3.5 and increased again to approximately 8 mg Fe/g protein at pH 3.0. Because the MPC was insoluble at pH 3.0 and 3.5, the added iron was unlikely to bind to the proteins and this caused an increase in the amount of iron in the supernatant. The large amount of free iron found in the permeate at pH 3.5 and 3.0 also showed that the soluble proteins in MPC at these pH values could not bind the soluble iron. Therefore, the amount of iron bound to the soluble proteins in MPC decreased at pH 3.5. The increase in the bound iron at pH 3.0 was perhaps due to inaccuracy in the calculation because the amount of proteins was very small.

In addition, Hekmat and McMahon (1998) studied the addition of iron to skim milk as a function of pH (from 6.7 to 4.0) using ultracentrifugation (52,000 *g*, 1 h). Electrophoresis of the soluble protein showed that the α_s-, β- and κ-casein bands were observed at pH 6.7–5.3 but no caseins bands were observed and more β-Lg had sedimented at pH $\leq$ 4.5. This could also explain the higher amount of iron bound to the soluble proteins in MPC between pH 7.0 and 5.5, which then decreased as the pH was lowered to 3.5 and 3.0.

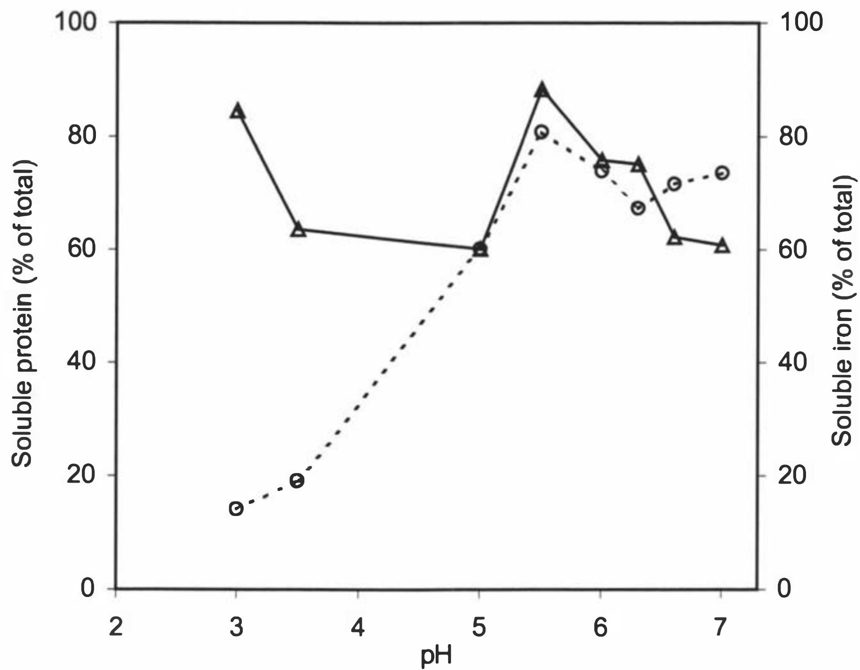


Figure 4.40. Effect of pH on the solubility of 1% MPC solution (o) and iron (△) in iron–MPC mixtures after addition of 2 mM ferrous sulphate (in 50 mM HEPES buffer).

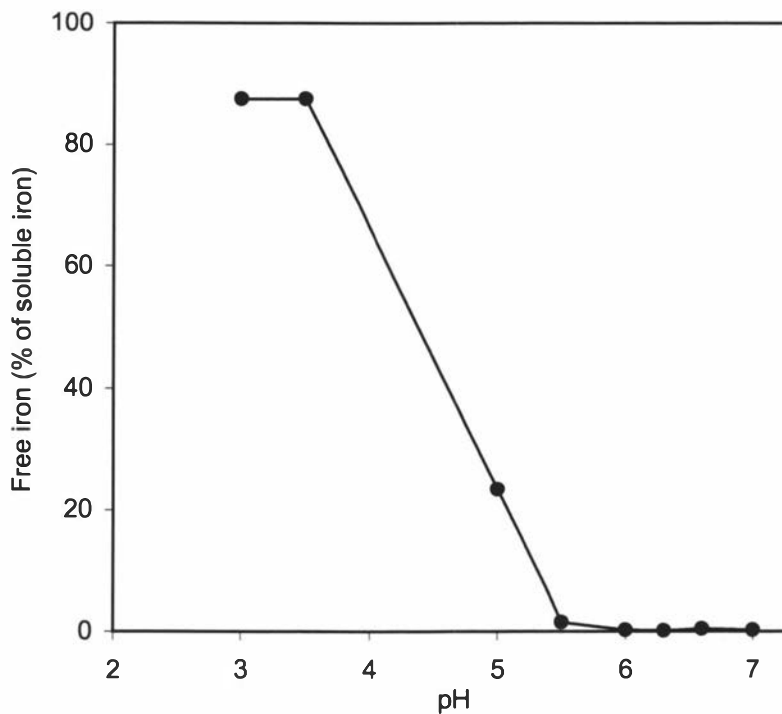


Figure 4.41. Effect of pH on the free iron (iron in the ultrafiltration permeate) in iron–MPC mixtures (in 50 mM HEPES buffer) (calculated as a percentage of the soluble iron in the supernatant). The total iron concentration in the original solution was 2 mM.

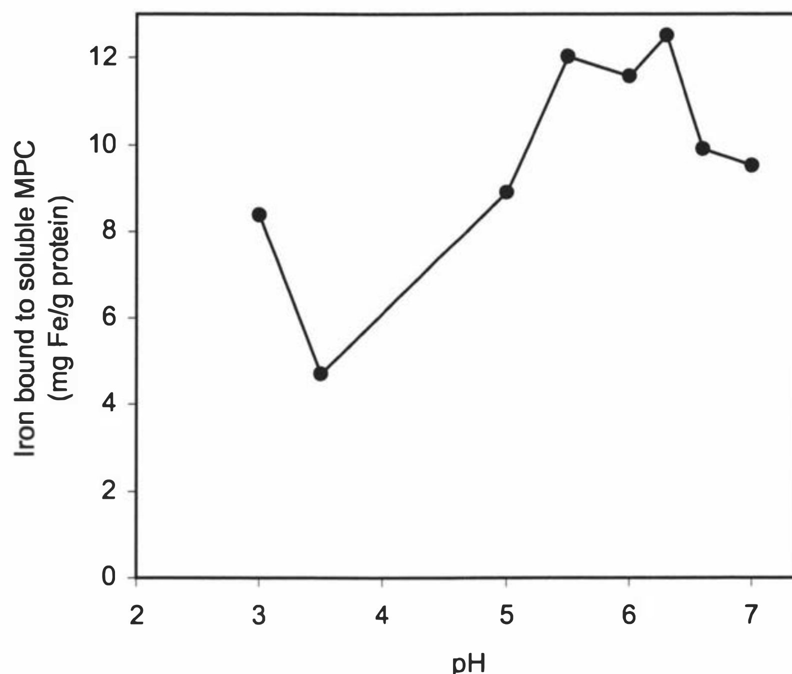


Figure 4.42. Effect of pH on the amount of iron bound to soluble MPC, in 50 mM HEPES buffer.

4.8.4. Effect of Ionic Strength on the Binding of Iron to MPC

An increase in the ionic strength of iron–MPC mixtures from 0.1 to 0.5 M by the addition of NaCl did not cause significant changes in the solubility of the proteins in the mixtures. The amount of free iron found in the ultrafiltration permeate also showed no significant changes as the ionic strength increased; nearly no free iron was found in the permeate. Therefore, the amount of iron bound to the soluble proteins in MPC was also not significantly affected. There was approximately 10 mg bound Fe/g MPC as the ionic strength of the mixtures was increased under the experimental conditions used in this study.

Table 4.4. Effect of ionic strength on the ability of MPC to bind iron in iron–MPC mixtures (containing 2 mM ferrous sulphate, in 50 mM HEPES buffer at pH 6.6)

Ionic strength (M)	Soluble protein (% of total)	'Free' iron (% of soluble iron)	Soluble iron bound to soluble MPC (mg Fe/g protein)
0.1	53.4	0.4	10.3
0.25	53.8	0.7	10.0
0.5	52.8	0.8	9.7

4.9. Discussion

Metal ions exhibit complex chemistry. One of the main considerations is the solubility of metal ions in aqueous solutions. Because iron is one of the transition metals that has more than one valence state (Labuza, 1971), its solubility is affected by a number of factors, such as pH and the conditions of the environment. The solubility of ferrous iron in aqueous solutions is quite high. However, ferrous iron readily oxidises to ferric iron in the presence of oxygen, and then forms precipitates with hydroxides. The rate of oxidation and consequently the extent of precipitation increase markedly with an increase in the solution pH. The insolubility of ferrous sulphate under the experimental conditions used in this study was due mainly to the formation of insoluble ferric hydroxide at relatively high pH. As expected, holding time was also found to enhance the formation of the iron hydroxides (Figure 4.3).

In the present study, it was found that addition of sodium caseinate to ferrous sulphate solution (pH 6.6) markedly improved its solubility (Figure 4.5). This increase in solubility of ferrous sulphate in the presence of sodium caseinate was due mainly to the binding of iron to the caseins, which consequently decreased the amount of iron that could react with hydroxides to form insoluble ferric hydroxide. In solutions containing 1% caseinate and up to 4 mM ferrous sulphate, virtually all the iron was bound to the protein molecules. This was indicated by very low levels of free iron in the

ultrafiltration permeates (Figure 4.13). Moreover, the qualitative cyanide assay showed that no colour reaction formed in the iron–casein complex (Table 4.1), suggesting that the iron added was bound completely to the caseins. It is unclear whether ferrous or ferric iron bound to the casein molecules. Manson and Cannon (1978) showed that caseins rapidly catalyse the oxidation of iron from the ferrous to the ferric state with the formation of stable ferric–phosphoprotein complexes.

In general, addition of iron to proteins involves binding of this cation to specific sites on the protein molecules. Casein is known to have the ability to bind several metal ions such as Ca^{2+}, Fe^{2+} and Zn^{2+} (Walstra and Jenness, 1984; Rollema, 1992). This ability is due mainly to the presence of clusters of phosphoserine residues in the casein molecules. The number of phosphoserine residues differs among individual casein fractions; α_{s1}- and α_{s2}-caseins have 13 and 8 phosphoserine residues per molecule, β-casein has 5 such residues and κ-casein has only 1 phosphoserine residue. As expected, the cation-binding ability of caseins follows the order: α_{s1}- and α_{s2}-caseins > β-casein > κ-casein.

The strong affinity of caseins for binding to iron has been recognised (Carmichael *et al*, 1975; Hegenauer *et al*, 1979a). This affinity is attributable to clustered phosphoserine residues in the caseins (Demott and Dincer, 1976; Manson and Cannon, 1978; Hegenauer *et al*, 1979a). In iron–casein complexes, the iron is probably bound to the caseins via the oxygen of the phosphate group and adopts a tetrahedral co-ordination structure (Webb *et al*, 1973). Apart from the phosphoserine residue as the main binding site for iron, there are other possible weak binding sites. For example, Reddy and Mahoney (1991a) found that enzymic dephosphorylation of α_{s1}-casein reduced the binding of iron but did not eliminate it. Hence, other possible binding sites that have been suggested are carboxyl groups (glutamic and aspartic residues), phenolic groups (tyrosyl residue), sulphydryl groups (cystenyl residue) and imidazole groups (hystidyl residue) (Gaucheron *et al*, 1996, 1997b).

Addition of iron at above a certain critical concentration to sodium caseinate causes aggregation of casein molecules, resulting in a loss of protein solubility (Figure 4.5). The aggregation and subsequent precipitation of sodium caseinate by the addition of iron probably occurs when all the binding sites are saturated. The high solubility of caseinate in water is due to the large number of negatively charged amino acid residues, with a major contribution from the phosphoserine clusters. The binding of iron to negatively charged residues will result in charge neutralisation, with a decrease in the electrostatic repulsions between the protein molecules. The decreased repulsions between protein molecules will also promote interactions between hydrophobic regions of the casein molecules. The formation of intermolecular bridging caused by the addition of iron may also be involved in the aggregation process (Gaucheron *et al*, 1996).

The role of electrostatic interactions in iron-induced aggregation is further confirmed by the observations on the effects of pH on the extent of casein aggregation and precipitation (Figure 4.6). The minimum amount of iron needed to initiate protein precipitation decreases with a decrease in the pH of the solution. Because of the lower overall negative charge at low pH values, a lower concentration of iron is required to saturate the binding sites and neutralise the negative charges.

Addition of iron to caseinate solution above the saturation binding level causes a gradual co-precipitation of the protein and iron. It is likely that the iron is still associated with the precipitated fraction of the casein. However, as the ferrous sulphate used in this experiment was not soluble under the experimental conditions, some of the iron may have precipitated by itself, through the formation of iron hydroxides.

Changes of pH in the range 3.0–7.0 affect the ability of the caseins in sodium caseinate to bind iron. When the pH was decreased from 7.0 to 5.0, there were no changes in the amount of iron bound to the caseins, in agreement with the results of Baumy and Brule (1988b) (the pH was changed from 5.0 to 8.0). However, lowering the pH from 5.0 to 3.0 resulted in a decrease in the amount of iron bound by caseins (Figure 4.20), which

disagreed with the results reported by Gaucheron *et al* (1996). These differences may have been due to the fact that, under the experimental conditions used in this study, sodium caseinate was not soluble at pH < 5.0 (Figure 4.19). This caused a decrease in the available caseins in the soluble fractions to bind the added iron at these pH values. Nelson and Potter (1979) showed that the uptake of both ferrous iron and ferric iron by soluble casein changed with a change in pH; a maximum iron uptake was observed between pH 6.0 and 7.0.

Changes in pH generally affect the complex formation between metal ions and proteins as hydrogen ions compete with the metal ion for binding to protein. At low pH, the reactive side chains of amino groups tend to become protonated, which decreases their affinity for cations, thus reducing their complexation with the protein. At low pH, there is a decrease in the ionisation of the phosphoserine groups and this supposedly causes a decrease in the cation binding by casein molecules on reducing the pH. However, Gaucheron *et al* (1996) stated that, because a change in pH did not affect the iron-binding ability of caseins, the iron is probably bound to caseins not only by ionic bonds with phosphoserine but also by co-ordination bonds. The possible co-ordination links are through NH_2, COOH, CONH and H_2O (Baumy and Brule, 1988b). When the pH is increased, the same side chains will acquire a negative charge and will tend to complex with cations. Hence, binding is generally higher at a more neutral pH. In addition, the change in the pH of the system can lead to reversible conformational changes in the proteins, thereby altering their metal-binding capacity. In the case of caseinate, a decrease in pH below 5.0 causes aggregation of casein molecules and consequently the caseins precipitate out of solution as the isoelectric point (pH 4.6) is approached.

Increasing the ionic strength from 0.1 to 0.5 M did not cause significant changes in the binding of iron to the proteins in sodium caseinate, in agreement with the findings of Gaucheron *et al* (1997a) and Baumy and Brule (1988b). Increasing ionic strength in the medium causes a competition in binding to the binding sites in caseins between the sodium ions and the iron ions. However, this competition might not happen because caseins have been reported to have better binding affinities for iron ions than for sodium

ions. Also, the binding ability of caseins for iron is not affected by a change in ionic strength because of the possible presence of co-ordination links.

α-La and β-Lg, the two major proteins that constitute WPI, have been shown to bind metal ions. Relatively little information on the binding properties of commercial whey protein products, such as WPI, is available. A review by Jackson and Lee (1992) states that α-La, immunoglobulins and lactoferrin are able to bind iron under certain conditions. α-La is a globular metalloprotein with a structure stabilised by four sulphur bridges and has four chelating sites for minerals (Kronman and Bracther, 1984). Murakami and Berliner (1983) found two binding sites for α-La; the first binds Ca^{2+} and the second, specific for Zn^{2+}, is also able to bind Co^{2+} and Cu^{2}. The ability of α-La and β-Lg to bind iron has been investigated by Baumy and Brule (1988a). They found that α-La can bind up to six ions of Fe^{2+} whereas the binding ability of β-Lg is > 3.5 ions of Fe^{2+} (at pH 6.6, ionic strength < 0.01 M). The binding sites on α-La and β-Lg for divalent cations are considered to consist of a group of ionic amino acids, such as carboxylic groups located on the side chain of glutamic and aspartic acids.

In contrast to the behaviour of sodium caseinate, where interactions of iron with caseins resulted in protein aggregation and precipitation of protein, the interactions of iron with the whey protein molecules in WPI did not cause significant precipitation of iron–WPI complexes (Figure 4.22). The aggregates of iron–WPI complexes were probably smaller in size or the extent of aggregation was much lower than that found in iron–sodium caseinate complexes.

Similar to caseinate, a change in pH of the WPI solution from 7.0 to 3.0 showed a marked decrease in the iron-binding ability of the whey proteins (Figure 4.31). This could have been due to changes in the ionic amino acids, which become more dissociated at low pH, hence reducing the ability of whey proteins to bind iron. Conformational changes in the whey proteins at low pH might also alter their ability to

bind iron. For example, it has been shown that β-Lg exists as a dimer between pH 7.5 and 5.5, and it forms octamers between pH 3.5 and 5.2 (deWit, 1989).

In WPI, increasing ionic strength caused a slight decrease in the amount of iron bound to the soluble whey proteins in WPI (Table 4.2). This is in general agreement with the studies on pure α-La and β-Lg, which show that the ability of these proteins to bind iron decreases with increasing ionic strength (Baumy and Brule, 1988a). An increase in ionic strength causes binding competition between sodium and the added iron in α-La and β-Lg, which decreases their binding ability. Also, changing the ionic strength might change the tertiary structure of the proteins, and hence a lower amount of iron is bound (Baumy and Brule, 1988a).

Analysis of the binding curves by Scatchard and Klotz plots showed that sodium caseinate has a higher binding affinity for iron (log K_{app} = 5.3) than WPI (log K_{app} = 3.6). Sodium caseinate also has a significantly higher number of binding sites (n = approximately 14) than WPI (n = approximately 8). The higher binding affinity of sodium caseinate is expected because caseins are known to have a stronger binding capacity than whey proteins for metal cations, mainly because of the presence of clusters of phosphoserine residues. The more compact structure of the whey proteins (i.e. globular structure) in WPI, compared with the more flexible, open structure of sodium caseinate, could also account for the differences in their ability to bind added iron.

MPC is a milk protein product that contains both caseins and whey proteins with similar properties to those of their native forms in skim milk. The caseins in MPC are thus in the form of micelles bound together by colloidal calcium phosphate (Munro, 2003). The MPC used in the present study comprised approximately 80% caseins and 20% whey proteins; this composition is very similar to that in normal milk.

There are no published reports on the iron-binding properties of MPC. However, in skim milk, a vast majority of added iron (about 85%) has been shown to bind to the

casein fractions (i.e. the micellar phase). A small proportion of iron is bound to the whey proteins and citrate (Carmichael *et al*, 1975; Gaucheron *et al*, 1997a; Hekmat and McMahon, 1998). Most of the iron added to cow's milk binds to the phosphoserine residues in the casein micelle, and some is sequestered by inorganic phosphate in the casein micelle (Jackson and Lee, 1992).

The results obtained in the present study showed that iron added to MPC was initially bound to the larger casein micelles. These large micelles normally contain relatively larger proportions of α_s- and β-caseins and have higher amounts of colloidal calcium phosphate (Pyne, 1962). With increasing addition of iron, binding also occurred with the small casein micelles. Smaller casein micelles have been shown to contain higher proportions of κ-casein and low amounts of colloidal calcium phosphate (Pyne, 1962). It was also found that addition of iron induced aggregation of the casein micelles, and the extent of aggregation increased with increasing iron concentration. This was probably due to the neutralisation of negative charges on the micelle surface, reducing the electrostatic repulsions between the micelles. Some of the iron may have also associated with the whey proteins because these proteins were still soluble after the addition of iron (Figure 4.36).

The binding sites for iron in MPC are likely to be similar to the binding sites found in caseinate, which are mainly the phosphoserine residues. However, the extent of iron binding on the basis of per gram of casein was much higher in MPC than in caseinate. For example, MPC could bind approximately 45 mg Fe/g protein in the soluble fraction, which was greater than the amount of iron that was bound to sodium caseinate (approximately 20.4 mg Fe/g protein) or WPI (approximately 19 mg Fe/g protein). The ability of MPC to bind a greater amount of iron because of the presence of both casein and whey protein fractions suggests that there were binding sites in the MPC other than the phosphoserine residues of caseins.

The nature of the binding of iron to caseins in MPC and in sodium caseinate is likely to be different because of the different state of the casein molecules in the milk protein

products. In sodium caseinate, the casein fractions are present as monomers and small complexes and iron binds mainly to the phosphoserine residues on the casein molecules. In contrast, the phosphoserine residues in MPC are not available as they interact with the calcium phosphate to form colloidal calcium phosphate. Iron is likely to be incorporated into the colloidal calcium phosphate, possibly displacing calcium from the phosphoserine residues and from the inorganic phosphate, forming iron phosphate. Thus, the iron in MPC is associated with both the caseins and the colloidal calcium phosphate.

It was found that a decrease in pH from 7.0 to 3.0 also caused a decrease in the amount of iron bound to the soluble protein fractions in MPC. This decrease was probably due to the insolubility of the protein in the MPC (in the absence of iron) at pH less than 5.0 (Figure 4.37). Dissolution of calcium and phosphate from the casein micelles at low pH has been reported (Pyne and McGann, 1960). Because, in the present study, the pH of the MPC solution was adjusted before the iron was added, it was likely that, when most of the casein micelles had aggregated (at pH < 5.0) and the colloidal calcium phosphate had dissolved, the iron added was not able to bind to the proteins. Also, because colloidal calcium phosphate solubilises at low pH, the structure of the micelles changed and this may have caused the decrease in the ability of the micelles to bind iron. At the pH where MPC was soluble (pH > 5.0), added iron was able to be bound by the soluble proteins and to be retained by the casein molecules (at pH 6.0–7.0).

Gaucheron *et al* (1997b) reported that there was no association of iron ions to colloidal calcium phosphate in skim milk because the iron was not released as free iron by ultrafiltration whereas both calcium and phosphate were released when the pH was decreased from 6.7 to 4.0. These results disagree with the findings in the present study. The differences may have been due to the different methodologies used in carrying out the experiment. Gaucheron *et al* (1997b) made up the iron–skim milk complexes initially before the pHs of the mixtures were changed. Once the iron was bound to the proteins in MPC, it might bind more strongly and the links that are formed between the iron ions and the protein molecules are probably more stable. As mentioned before, in

the present study, the pH of the MPC solution was adjusted before the iron was added. The changes in the casein molecules in MPC when the pH was decreased may have affected their ability to bind iron, and hence the difference in the results.

Increasing ionic strength from 0.1 to 0.5 M did not cause significant changes in the binding of iron to the proteins in MPC. Similar to sodium caseinate, the casein molecules that were responsible for the binding of iron in this milk protein were shown to have better binding affinities for iron ions than for sodium ions. Also, the links between the iron ions and the colloidal calcium phosphate in MPC are probably more stable and hence binding was not affected by a change in ionic strength (Gaucheron *et al*, 1997a; Baumy and Brule, 1988b).

CHAPTER 5
BINDING OF ZINC TO MILK PROTEIN PRODUCTS

5.1. Introduction

It is well known that milk proteins have the ability to bind several metal ions including zinc. The association of zinc in cow's and human milk has been previously studied; 95% of the zinc in skim milk was found to associate with the casein micelles (Blakeborough *et al*, 1983; Singh *et al*, 1989b). A small proportion of the zinc in both cow's milk and human milk was associated with low molecular weight compounds (Cousins and Smith, 1980; Martin *et al*, 1981; Blakeborough *et al*, 1983; Martin *et al*, 1984; Singh *et al*, 1989b), which have been identified as citric acid and picolinic acid (Martin *et al*, 1981). Cousins and Smith (1980) reported that this association occurs only after binding sites of higher molecular weight ligands become saturated with zinc.

Previous studies have generally contended that the casein fraction has a higher binding affinity for zinc than the whey protein fraction (Cousins and Smith, 1980; Harzer and Kauer, 1982; Blakeborough *et al*, 1983; Singh *et al*, 1989a). Thus, several studies have been carried out on the binding of zinc to caseins in various forms: bovine and human casein (Singh *et al*, 1989a), purified individual caseins (Baumy and Brule, 1988b; Singh *et al*, 1989a) and commercial sodium caseinate (Gaucheron *et al*, 1997a). Studies on the addition of zinc to whey proteins have been carried out mainly on the binding of zinc to individual whey proteins, such as α-La, β-Lg and BSA (Kronman *et al*, 1981; Baumy and Brule, 1988a; Kronman, 1989; Singh *et al*, 1989a; Ren *et al*, 1993).

The characteristics of the binding of zinc to different milk proteins (either caseins or whey proteins) depend on the structure of the protein and the ability of the protein to bind the added zinc. The extent of the binding of zinc by milk proteins is also affected

by different environmental conditions, such as pH, ionic strength, temperature and reaction time.

The binding of zinc to caseins has been attributed to the negatively charged phosphoserine residue in caseins (Harzer and Kauer, 1982; Baumy and Brule, 1988b; Singh *et al*, 1989a; Gaucheron *et al*, 1997a). There are also other possible binding sites for zinc, such as carboxylic groups or histidine (Singh *et al*, 1989a). The binding of zinc to caseins causes changes in the protein structure and causes protein aggregation. Precipitation of caseins has been reported to occur at a critical zinc concentration, when the binding sites in the casein become saturated (Baumy and Brule, 1988b; Singh *et al*, 1989a; Gaucheron *et al*, 1997a). In general, whey proteins show little capacity for zinc binding compared with caseins. However, α-La has been reported to have the ability to bind added zinc (Kronman *et al*, 1981; Baumy and Brule, 1988a; Kronman, 1989; Singh *et al*, 1989a; Ren *et al*, 1993).

As mentioned above, thus far, studies on the binding of zinc have focused mainly on the addition of zinc to milk, individual casein fractions, individual whey protein fractions and laboratory based protein preparations. No systematic studies have been carried out on the binding of zinc by commercial milk protein products. Hence, the objective of this part of the work was to characterise the binding of zinc to different commercial milk protein products – sodium caseinate, whey protein isolate (WPI) and milk protein concentrate (MPC) – and to determine how this binding is affected by different environmental factors.

5.2. Solubility of Zinc Sulphate in HEPES buffer

Various amounts of zinc sulphate were dissolved in 50 mM HEPES buffer (pH 6.6) and the solutions were centrifuged at 10,800 *g* for 20 min. The solubility was defined as the amount of zinc in the supernatant as a percentage of the total added zinc. Under these conditions, the solubility of zinc decreased with increasing zinc concentration.

About 90% of the zinc was soluble at up to 3 mM added zinc sulphate (Figure 5.1). As the added zinc sulphate concentration was increased, the solubility decreased and the solubility levelled off at approximately 70% at above 6 mM added zinc.

The decrease in the solubility of zinc sulphate may have been due to the formation of zinc hydroxide. These zinc hydroxides are reported to precipitate from solutions of salts by the addition of bases (Cotton *et al*, 1987). Hence, when the pH was adjusted to 6.6, the formation of zinc hydroxide according to equation (1) was likely to occur.

$$Zn^{2+} + 2OH^{-} \leftrightarrow Zn(OH)_2 \quad (1)$$

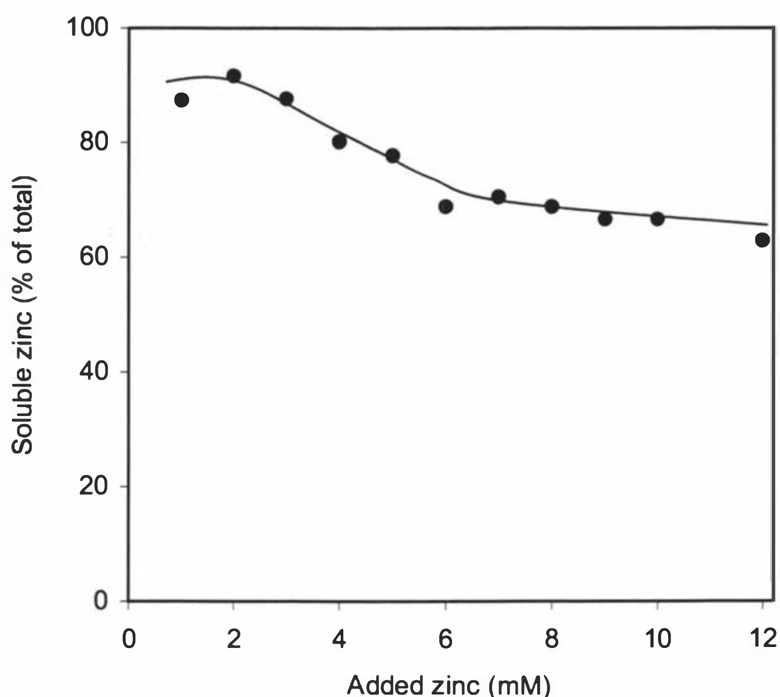


Figure 5.1. Solubility of zinc sulphate in 50 mM HEPES buffer, pH 6.6.

5.3. Solubility of Zinc and Sodium Caseinate in Zinc Sulphate–Sodium Caseinate Mixtures

The solubility of zinc (in HEPES buffer, pH 6.6) in the presence of sodium caseinate was different from the solubility of zinc sulphate alone. In the presence of sodium caseinate, the zinc was about 90% soluble at up to 2 mM added zinc sulphate, and its solubility decreased gradually at between 3 and 5 mM added zinc sulphate (Figure 5.2). Samples containing > 5 mM added zinc sulphate had only 20% solubility (i.e. approximately 80% of the added zinc had precipitated).

When the solubility of 1% sodium caseinate solution (in HEPES buffer, pH 6.6) without addition of zinc sulphate was investigated under the same conditions, it was found that sodium caseinate was more than 90% soluble. When zinc was added to the caseinate solution, the sodium caseinate was almost fully soluble (> 90%), up to an added zinc concentration of approximately 2.5 mM (Figure 5.3). Further addition of zinc caused a considerable decrease in the solubility of sodium caseinate. At ≥ 6 mM added zinc sulphate, most of the sodium caseinate had precipitated out of the solution.

The aggregation of zinc–sodium caseinate mixtures was measured using turbidity measurements at 650 nm as a function of the added zinc concentration before and after centrifugation (Figure 5.4). The turbidity of zinc–sodium caseinate mixtures increased markedly at > 2 mM added zinc, indicating that addition of zinc at above this concentration caused the formation of large protein aggregates.

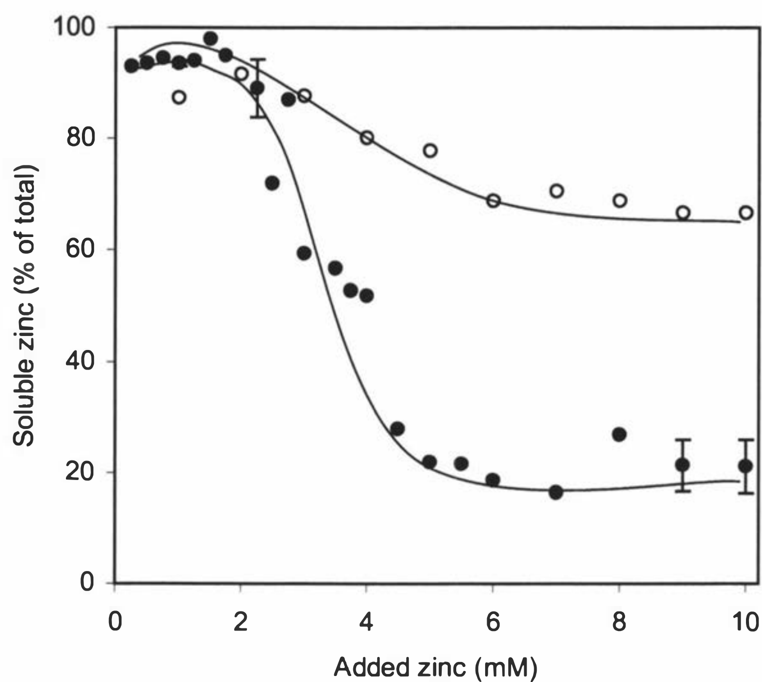


Figure 5.2. Solubility of zinc in the absence (o) and in the presence (•) of sodium caseinate, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

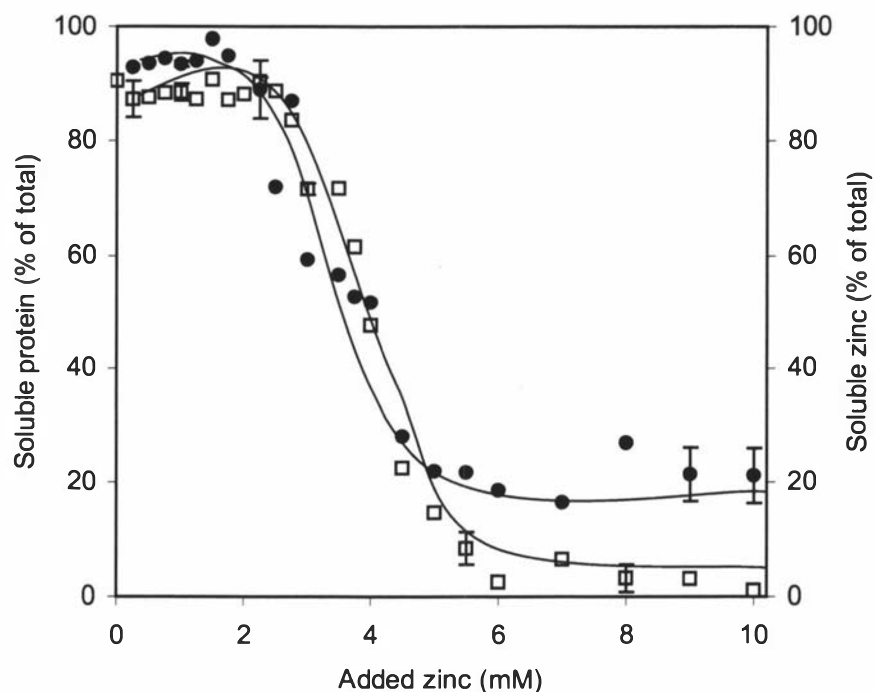


Figure 5.3. Solubility of zinc (•) and sodium caseinate (□) in zinc sulphate–sodium caseinate mixtures, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

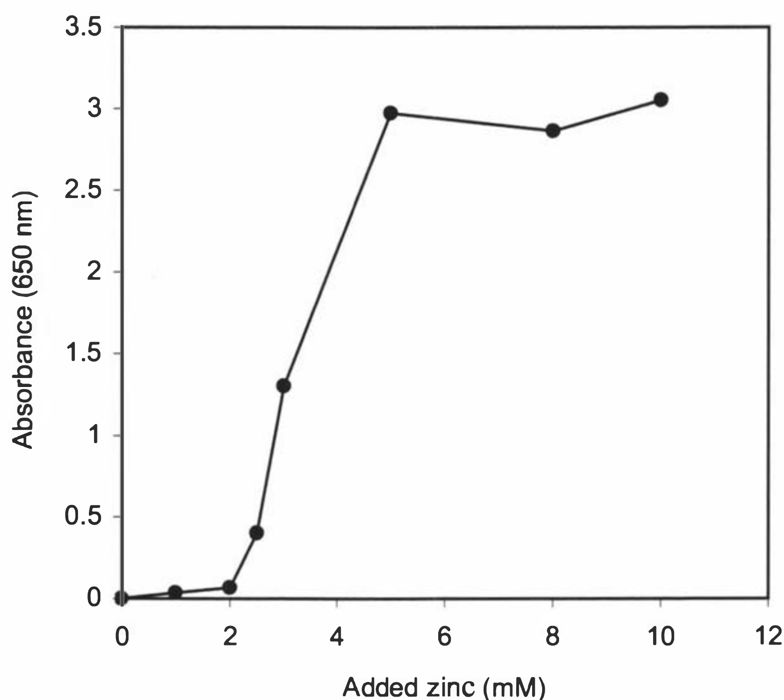


Figure 5.4. Turbidity (at 650 nm) of zinc sulphate–sodium caseinate mixtures as a function of added zinc concentration.

As shown in Figure 5.3, the solubility profiles of zinc sulphate and sodium caseinate in the mixture followed a fairly similar pattern. Both zinc and sodium caseinate began to precipitate out of the solution (i.e. decrease in solubility) at above 2.5 mM added zinc. Because zinc in the absence of sodium caseinate was soluble under these experimental conditions (Figure 5.1), it can be postulated that the added zinc had associated with the sodium caseinate and that they then co-precipitated. The minimum concentration of zinc required to cause precipitation of sodium caseinate was found to be 2.5 mM.

Precipitation of sodium caseinate as a result of the addition of zinc has been reported previously by Harzer and Kauer (1982) and Gaucheron *et al* (1997b). They reported that precipitation occurred when a sodium caseinate solution (5 mg protein/mL) was dialysed against buffers containing more than 50 μg zinc sulphate/mL (10 mg Zn/g protein) (Harzer and Kauer, 1982) or by addition of 6 mM zinc chloride (caseinate concentration 25 g/L), which corresponds to 15.7 mg Zn/g protein (Gaucheron *et al*,

1997b). In the present study, the precipitation of caseinate was found to occur at 16.35 mg Zn/g protein, which is essentially similar to the values reported by Gaucheron *et al* (1997b). The lower zinc concentration value obtained by Harzer and Kauer (1982) compared with the present study was probably due to the use of a different buffer and pH (0.1 N Tris/HCl buffer at pH 7.4 compared with 50 mM HEPES buffer at pH 6.6 in the present study).

5.3.1. Solubility of Individual Caseins in Zinc Sulphate–Sodium Caseinate Mixtures

Mixtures containing 1% sodium caseinate and various levels of zinc sulphate were centrifuged and the supernatants were analysed using SDS-PAGE.

As shown in Figure 5.5, the intensities of the α_s- (α_{s1}- + α_{s2}-), β- and κ-casein bands were not affected by the addition of up to 2 mM zinc sulphate. However, these bands showed a slight decrease in intensity at approximately 3 mM added zinc. The intensity of all casein bands diminished markedly at ≥ 3 mM added zinc and no clear bands could be seen in the gel at above 4 mM added zinc.

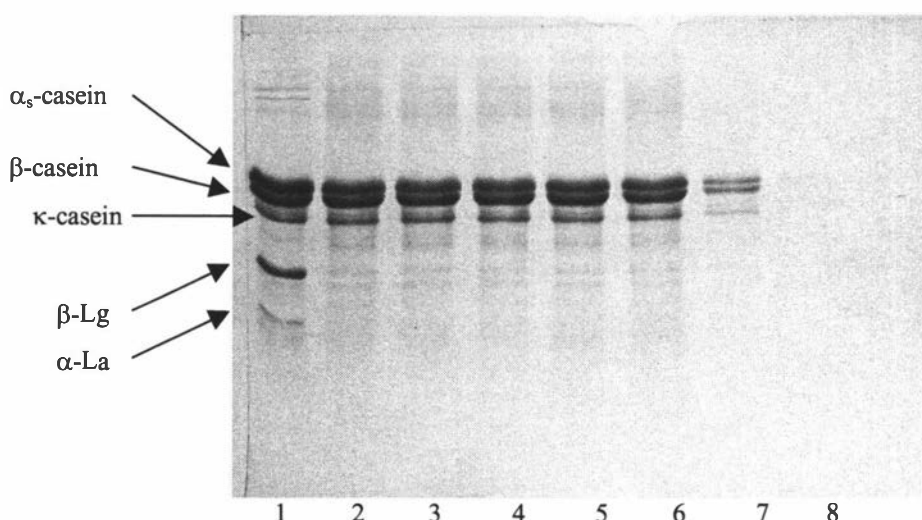


Figure 5.5. SDS-PAGE patterns of: skim milk (1), sodium caseinate solution without addition of zinc before centrifugation (2), the supernatant obtained after centrifugation (3), and zinc sulphate–sodium caseinate mixtures after centrifugation with added zinc at 1 mM (4), 2 mM (5), 3 mM (6), 4 mM (7) and 5 mM (8). Samples were dissolved in 50 mM HEPES buffer at pH 6.6.

5.4. Binding of Zinc to Sodium Caseinate

Because the addition of zinc at above certain concentrations to sodium caseinate solutions caused the precipitation of sodium caseinate (Figure 5.3), the zinc bound to sodium caseinate could be separated into two fractions: *zinc bound to soluble protein* and *zinc bound to insoluble protein*. The proportions of zinc in both these fractions were calculated based on the *total bound zinc*, as explained for iron in Section 4.4. The distribution of the total bound zinc between the soluble and insoluble fractions of protein is shown in Figure 5.6.

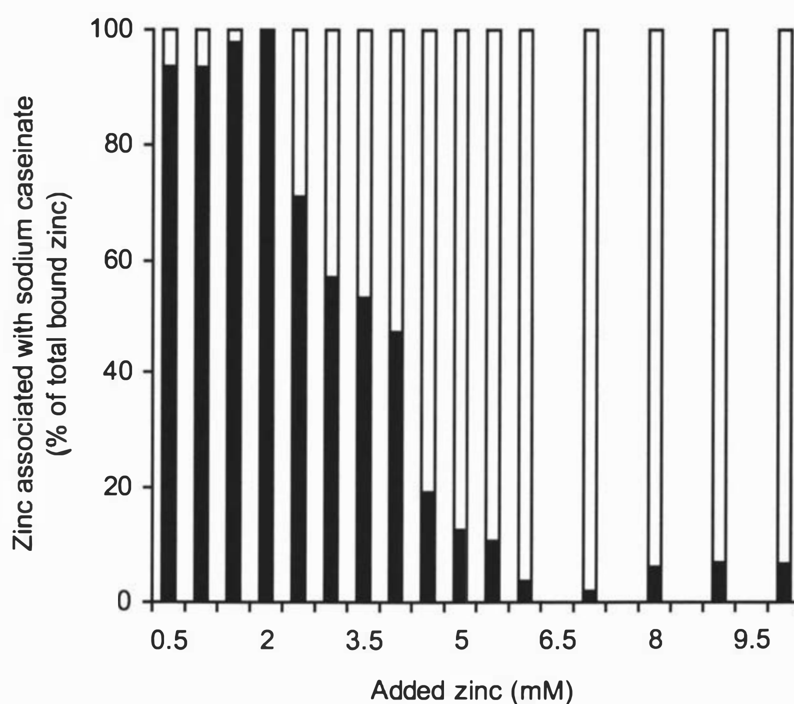


Figure 5.6. Distribution of bound zinc (as a percentage of the total bound zinc) between the soluble fraction (■) and the insoluble fraction (□) of sodium caseinate dissolved in 50 mM HEPES buffer at pH 6.6.

Figure 5.6 shows that addition of up to 2 mM zinc sulphate to sodium caseinate solutions resulted in > 95% of the total zinc being bound to the soluble protein fraction. With increasing amounts of added zinc, the relative proportions of zinc bound to soluble

protein decreased. At > 6 mM added zinc, less than approximately 10% of the total zinc was bound to soluble protein and the remainder had sedimented along with the sodium caseinate. The reason for the decreasing amount of soluble bound zinc was that sodium caseinate precipitated out of the solution at > 2.5 mM added zinc. Hence, it appears that the binding of zinc to soluble sodium caseinate was limited by the amount of zinc that could be added before precipitation of sodium caseinate occurred.

5.4.1. Binding of Zinc to Sodium Caseinate in the Soluble Fraction

When the binding in the soluble region (≤ 2.5 mM added zinc) was investigated in more detail using ultrafiltration, it was found that the amount of zinc in the ultrafiltration permeate (i.e. free zinc) was close to zero (Figure 5.7).

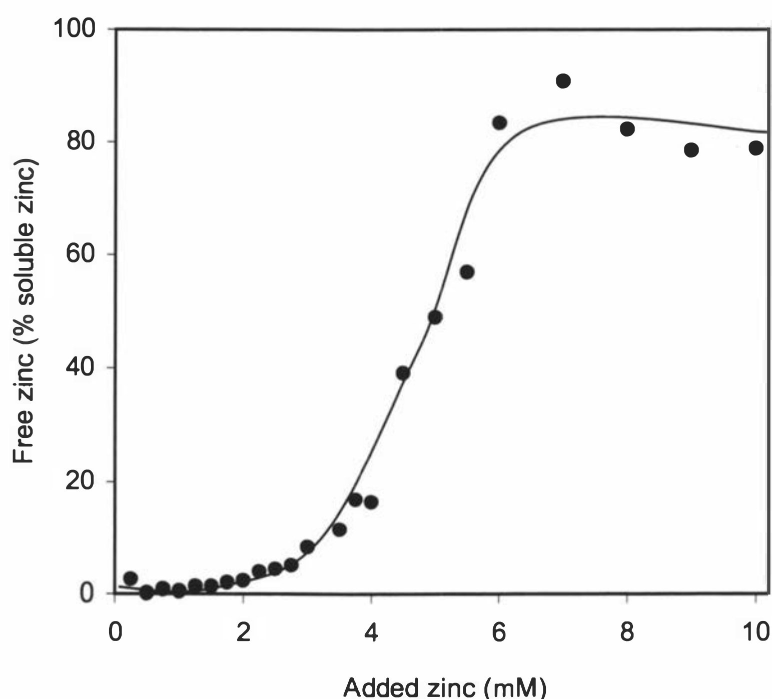


Figure 5.7. Amount of 'free' soluble zinc (i.e. in the ultrafiltration permeate) after ultrafiltration of zinc sulphate–sodium caseinate mixtures, in 50 mM HEPES buffer at pH 6.6.

This suggests that, under these conditions, most of the zinc was bound to sodium caseinate. This is in agreement with the results of Gaucheron *et al* (1997a), who reported no zinc in the ultrafiltration permeate (MW cut-off 25,000 Da) at 1.5 mM added zinc chloride concentration, when no casein precipitation was observed (final casein concentration 25 g/L).

The binding isotherm for the binding of zinc to sodium caseinate, in terms of mol Zn bound/mol protein, using an MW of 22,000 Da for caseinate, is shown in Figure 5.8. The binding of zinc to caseinate increased markedly with increasing free zinc concentration, and then levelled off. It was found that the caseinate was able to bind up to 5 moles of zinc per mole of protein before the protein began to precipitate.

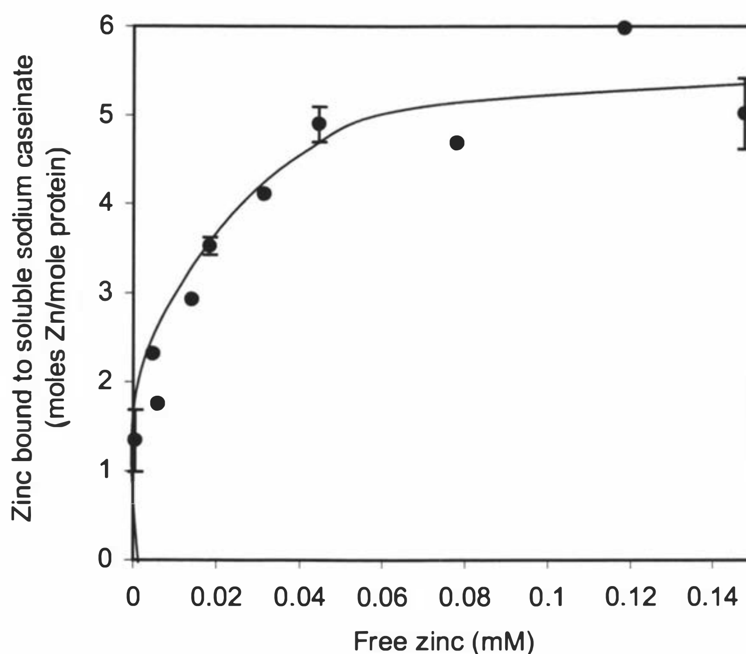


Figure 5.8. Binding of zinc to soluble sodium caseinate, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

As shown in Figure 5.3, the solubility of sodium caseinate decreased markedly at approximately 3–6 mM added zinc and little soluble sodium caseinate was left to bind the added zinc at above 6 mM. As a result, the amount of free zinc in the ultrafiltration permeate increased at above 3 mM added zinc and over 90% of the zinc was found as free zinc in the ultrafiltration permeate at above 6 mM added zinc (Figure 5.7). The binding isotherm was not quantified in this region because of the precipitation of sodium caseinate, which resulted in very low concentrations of soluble protein. Hence, it was suggested that a critical concentration of zinc was required for precipitation, which occurred when the protein bound approximately 5 moles of zinc per mole of protein.

5.4.2. Binding of Zinc to Sodium Caseinate in the Insoluble Fraction

The solubility profile of zinc in zinc sulphate–sodium caseinate mixtures followed the same trend as the solubility profile of sodium caseinate (Figure 5.3). It is postulated that binding of zinc caused precipitation of sodium caseinate. Therefore, it is likely that a certain amount of zinc will remain bound to the protein in the precipitate. The amount of zinc bound within the insoluble fraction was determined indirectly, i.e. by subtracting the amount of zinc in the supernatant from the total added zinc. It could be estimated that > approximately 90% of the added zinc was bound to the insoluble fraction of sodium caseinate (Figure 5.6) at > 6 mM added zinc sulphate (when most of the sodium caseinate had precipitated).

5.4.3. Binding Sites and Binding Constants

The binding data were analysed using the Scatchard plot to evaluate the apparent average association constant (K_{app}) and the maximum number of binding sites (n), as mentioned in Section 4.4.3.

The Scatchard plot for the binding of zinc to sodium caseinate is shown in Figure 5.9. The plot could be fitted to two straight lines. This indicates that there was more than

one class of binding sites, but that one binding site was preferentially filled. The first linear portion had a higher affinity (i.e. the slope of the linear portion was higher) than the second linear portion. The estimated number of high affinity binding sites (n) was approximately 6 and the value of log K_{app} was 4.84. This value was similar to the results of Singh *et al* (1989a), who found that bovine sodium caseinate had 6 binding sites for zinc and a log K_{app} value of 3.2. The second linear portion (II – Figure 5.9) indicates the presence of non-specific binding sites in the caseinate for zinc. Baumy and Brule (1988b) used Scatchard plots to analyse the binding of zinc to β-casein and showed the presence of two kinds of binding site; the first 5 sites showed a higher binding affinity than the 2 following sites. They suggested that the possible binding sites were probably composed of 5 phosphoserines and 2 carboxylic groups.

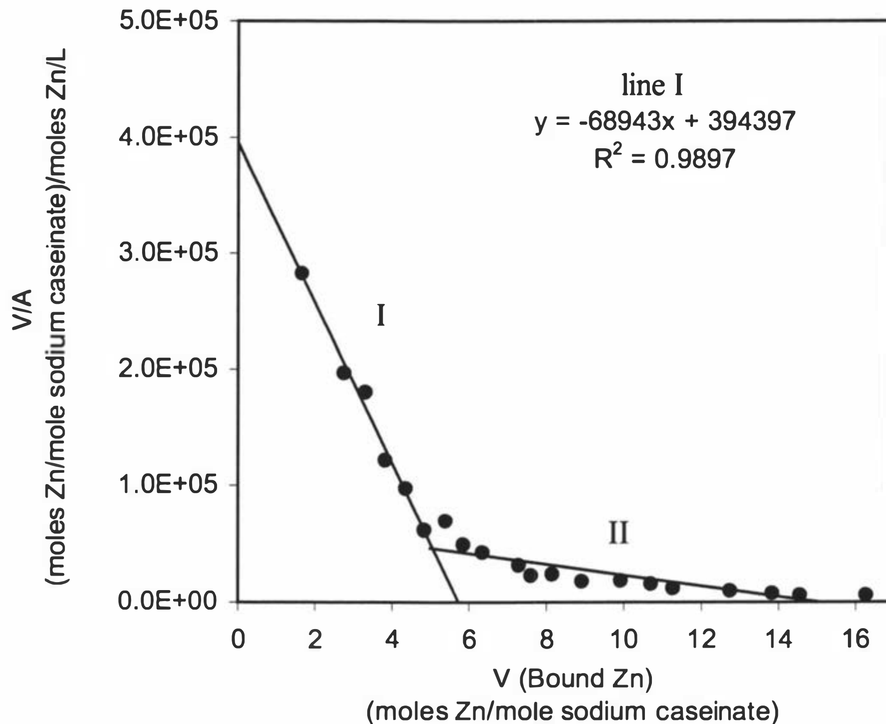


Figure 5.9. Scatchard plot for the binding of zinc to sodium caseinate, in 50 mM HEPES buffer at pH 6.6. All data points in line I were used in the regression analysis.

5.4.4. Effect of pH on the Binding of Zinc to Sodium Caseinate

The effect of pH on the binding of zinc by sodium caseinate was investigated at a zinc concentration of 2 mM, because the sodium caseinate could bind almost all the added zinc without precipitation at this concentration. Sodium caseinate solution (1% w/w protein in HEPES buffer) was adjusted to pH in the range 2.5–7.0. These solutions were incubated at 4°C overnight and then 2 mM zinc sulphate was added. The binding study was then carried out as described in the materials and methods section.

Acidification of the sodium caseinate solution before the addition of zinc affected the appearance and solubility of the protein (Figure 5.10). When the pH of the sodium caseinate solution was adjusted to 5.5 or 5.0, it became turbid. However, with overnight stirring and incubation, obvious precipitation was not observed. At pH 3.0 and 2.5, protein precipitation and sedimentation could be observed and the aggregates became smaller but the sedimentation did not disappear with overnight stirring and incubation.

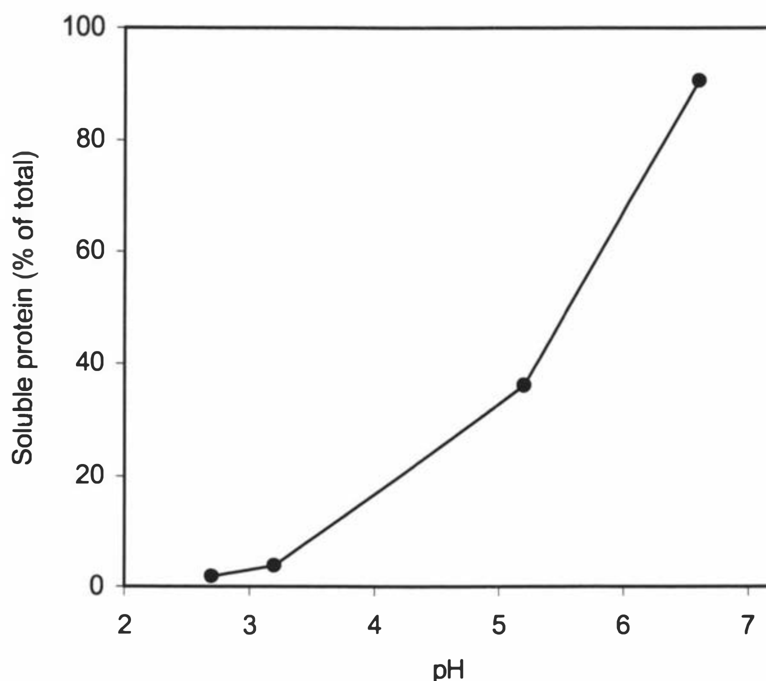


Figure 5.10. Effect of pH on the solubility of 1% sodium caseinate in 50 mM HEPES buffer with no added zinc sulphate.

Addition of 2 mM zinc had a marked effect on the solubility of sodium caseinate in the zinc–sodium caseinate mixtures in the pH range 2.5–5.5 under the conditions used in the experiment (Figure 5.11). The solubility of the added zinc (measured as the percentage of zinc that remained in the supernatant) was also affected by pH changes. Between pH 6.0 and 7.0, the solubility of sodium caseinate in the complex was not much affected; approximately 80% of the protein was still soluble and most of the added zinc was also recovered in the supernatant. A decrease in the pH to 5.5 caused a marked decrease in the solubility of the sodium caseinate. Below pH 5.5, the solubility of the sodium caseinate remained very low at around 10%. Only approximately 30% of the added zinc was soluble at pH 5.5 and 5.0, but most of the added zinc (> 80%) remained in the supernatant at pH 2.0 and 3.5 although most of the sodium caseinate had precipitated. Hence, it is probable that, at pH 5.0 and 5.5, caseinate was able to bind the added zinc, which then co-precipitated with the proteins on centrifugation. This did not occur at pH 3.0 and 2.5 because only a small amount of the added zinc could be found co-precipitating with the sodium caseinate. The reasons for the insolubility of sodium caseinate at low pH are discussed in Section 4.4.4.

A decrease in the pH also resulted in an increase in the amount of soluble zinc in the ultrafiltration permeate (i.e. free zinc). This can be seen from Figure 5.12, where the proportion of free zinc was approximately 0–5% at pH 6.0–7.0, but > 90% of the zinc was found in the permeate at pH ≤ 5.5. This showed that the binding of zinc to sodium caseinate decreased markedly when the pH was decreased to ≤ 5.0.

The free zinc versus pH profile (Figure 5.12) appeared to be the reverse of the profile of the sodium caseinate solubility as a function of pH (Figure 5.11). Because > 90% of the sodium caseinate was insoluble at below pH 6 (Figure 5.11), there was little soluble sodium caseinate left to bind the zinc ions in the mixture. As a result, there was a large increase in the amount of zinc in the ultrafiltration permeate at pH ≤ 5.5 (Figure 5.12). Hence, the precipitation of sodium caseinate during acidification reduced its ability to bind zinc (Figure 5.13).

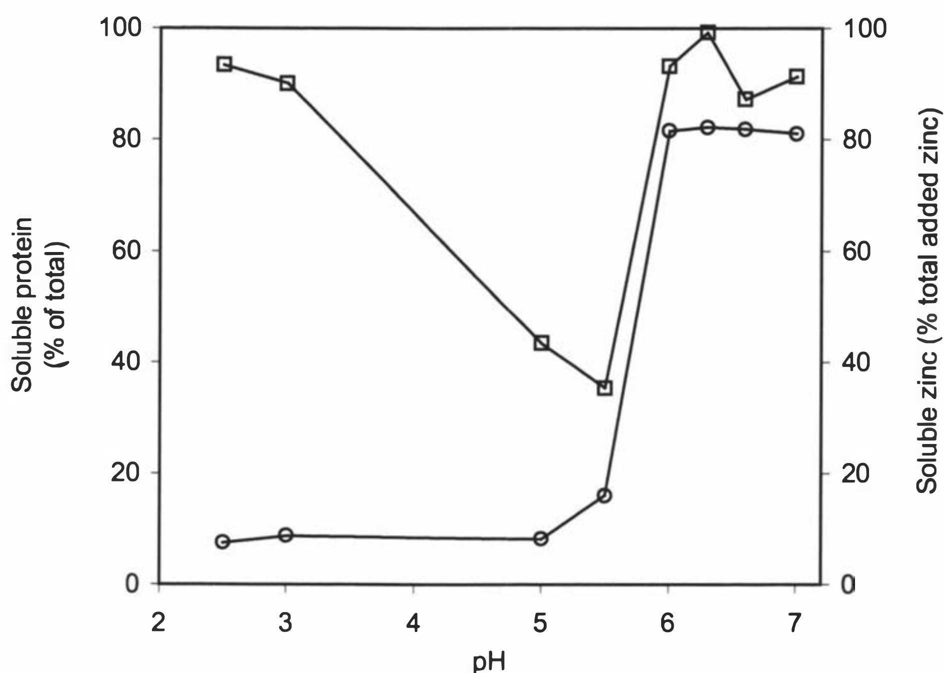


Figure 5.11. Effect of pH on the solubility of sodium caseinate (o) and zinc (□) in zinc sulphate–sodium caseinate mixtures (1% protein with 2 mM zinc sulphate in 50 mM HEPES buffer).

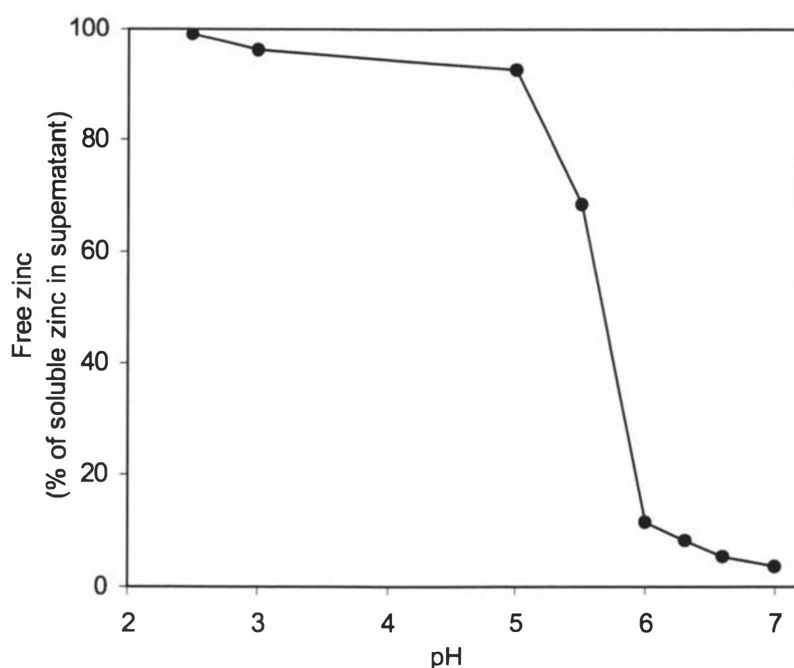


Figure 5.12. Effect of the pH of zinc sulphate–sodium caseinate mixtures (in 50 mM HEPES buffer) on the amount of free zinc in the ultrafiltration permeate (calculated as a percentage of the soluble zinc in the supernatant). The total concentration of zinc in the original solution was 2 mM.

Approximately 5–5.5 moles of zinc bound per mole of protein at pH 6.0–7.0 and this value decreased markedly to < 1 mole Zn/mole protein as the pH was lowered to 2.5.

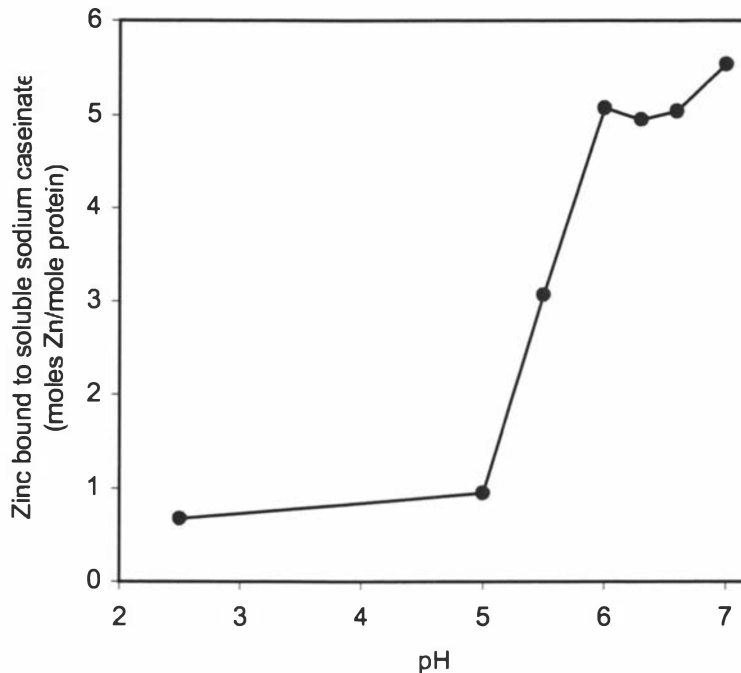


Figure 5.13. Amount of zinc bound to soluble sodium caseinate as affected by pH, in 50 mM HEPES buffer.

Acidification of casein solutions has been known to affect the ability of individual casein molecules (Baumy and Brule, 1988b; Singh *et al*, 1989a) and caseinate to bind zinc (Harzer and Kauer, 1982; Gaucheron *et al*, 1997a). Some researchers investigated the binding only in the pH range > 5.0 because precipitation of sodium caseinate occurred below pH 5.0. Baumy and Brule (1988b) investigated the binding of zinc chloride to β-casein (the β-casein was dissolved in water with no added NaCl in the pH range from 5.0 to 8.0). Singh *et al* (1989a) investigated the binding of zinc chloride to α_{s1}-casein in the pH range 5.0–7.0 because bovine α_{s1}-casein precipitated at pH values below 5.0, and precipitation of zinc occurred at pH 7.8.

Harzer and Kauer (1982) and Gaucheron *et al* (1997a) investigated the binding of zinc to casein at low pH (below pH 5.0). Harzer and Kauer (1982) reported complete protein precipitation and total release of zinc into the supernatant when the zinc–casein complex was acidified to pH 4.6 (in 0.1 N Tris/HCl buffer, pH 7.4, containing 1% NaCl). At pH 2.0, the casein was only slightly soluble and there was no binding of zinc to casein. The range of pH used by Gaucheron *et al* (1997a) was from 6.8 to 2.5. Acidification of casein (2.5% caseinate dissolved in water with no NaCl present) containing 1.5 mM zinc caused a gradual release of zinc ions into the ultrafiltration permeate. When the pH was 6.8, no zinc was found in the ultrafiltration permeate. The percentage of zinc ions being released started to increase at pH < 5.5 and there was a total release of zinc ions into the permeate at pH 2.5. Hence, there was a decrease in the binding of zinc by casein as the pH was lowered.

Overall, the trend of the results obtained in the present study on the effect of acidification on the binding of zinc to sodium caseinate was in accordance with the results obtained by other researchers (Harzer and Kauer, 1982; Baumy and Brule, 1988b; Singh *et al*, 1989a; Gaucheron *et al*, 1997a). It was found that the ability of sodium caseinate to bind zinc decreased when the pH was lowered.

5.4.5. Effect of Ionic Strength on the Binding of Zinc to Sodium Caseinate

An increase in NaCl concentration from 0.1 to 0.5 M in zinc sulphate–sodium caseinates mixture containing 2 mM added zinc sulphate (pH 6.6) decreased the solubility of sodium caseinate slightly (Table 5.1). An opposite trend in the percentage of free zinc in the ultrafiltration permeate was observed. The amount of free zinc increased slightly when the ionic strength was increased from 0.25 to 0.5 M. This increase may have been due to slight precipitation of sodium caseinate. As a result, the amount of soluble zinc bound to soluble sodium caseinate was not significantly affected by changes in the ionic strength from 0.1 to 0.5 M (Table 5.1).

Table 5.1. Effect of ionic strength on the ability of sodium caseinate to bind zinc in zinc sulphate–sodium caseinate mixtures (containing 2 mM zinc sulphate in 50 mM HEPES buffer at pH 6.6)

Ionic strength (M)	Soluble protein (% of total)	'Free' zinc (% of soluble zinc)	Soluble zinc bound to soluble sodium caseinate (mg Zn/g protein)
0.1	82.8	4.9	10.9
0.25	81.4	8.4	11.1
0.5	75.3	11.2	11.2

The effect of ionic strength on the binding of zinc to individual caseins has been investigated previously. Singh *et al* (1989a) investigated the effect of ionic strength (0–0.5 M NaCl) on the binding of zinc to α_{s1}-casein and found that increasing the ionic strength of the medium reduced the binding of zinc (from approximately 4.5 to 3 atoms of zinc per mole of protein). Baumy and Brule (1988b) also reported that the amount of zinc bound to β-casein decreased when the ionic strength was increased in the range 0, 0.05 and 0.1 M.

Gaucheron *et al* (1997a) investigated the effect of ionic strength on the binding of zinc to sodium caseinate and found that increasing the NaCl concentration in the range from 0 to 0.12 M did not cause any effect on the binding of zinc to caseins.

The results from the present study did not show significant changes in the zinc-binding ability of sodium caseinate with increasing ionic strength (from 0.1 to 0.5 M NaCl). Because ionic strength < 0.1 M NaCl was not investigated in the present study, the results of the present study cannot be compared directly with the results from Gaucheron *et al* (1997a).

5.5. Solubility of Zinc and WPI in Zinc Sulphate–WPI Mixtures

The solubility of zinc sulphate in the presence of WPI is plotted in Figure 5.14. Most of the added zinc was soluble (> 90%) up to 3 mM added zinc. Between 2 and 5 mM added zinc, the solubility decreased gradually and then leveled off at approximately 60% when the added zinc concentration was > 5 mM. When compared with the solubility of zinc sulphate alone (Figure 5.1), the presence of WPI in the zinc–WPI mixture appeared to cause a slight decrease in the solubility of zinc sulphate, especially at high added zinc concentrations.

Addition of zinc sulphate up to 4 mM to the zinc–WPI mixture had no effect on the solubility of WPI (approximately 90% of the total protein was soluble) (Figure 5.15). However, the solubility of the WPI decreased almost linearly with added zinc at > 4 mM zinc sulphate; only approximately 40% of the total WPI was soluble at 10 mM added zinc.

Protein aggregation in zinc–WPI mixtures as a function of the added zinc concentration was estimated using turbidity measurement at 650 nm (Figure 5.16). The turbidity increased with increasing zinc concentration. After centrifugation, the turbidity increased up to 4 mM added zinc, but decreased and remained very low above this concentration. This indicates that some of the protein aggregates had sedimented upon centrifugation.

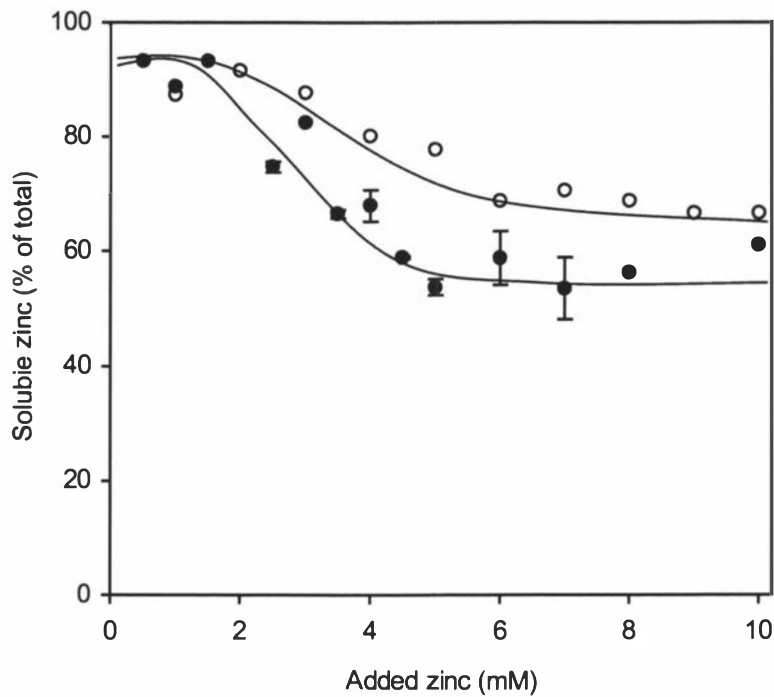


Figure 5.14. Solubility of zinc in the absence (o) and in the presence (•) of WPI, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

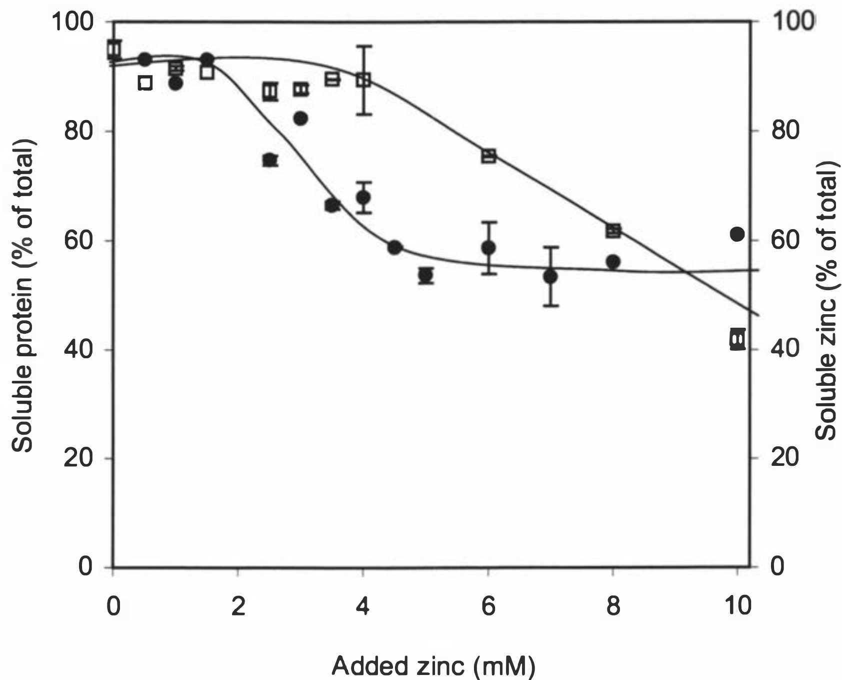


Figure 5.15. Solubility of zinc sulphate (•) and WPI (□) in zinc–WPI mixtures, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

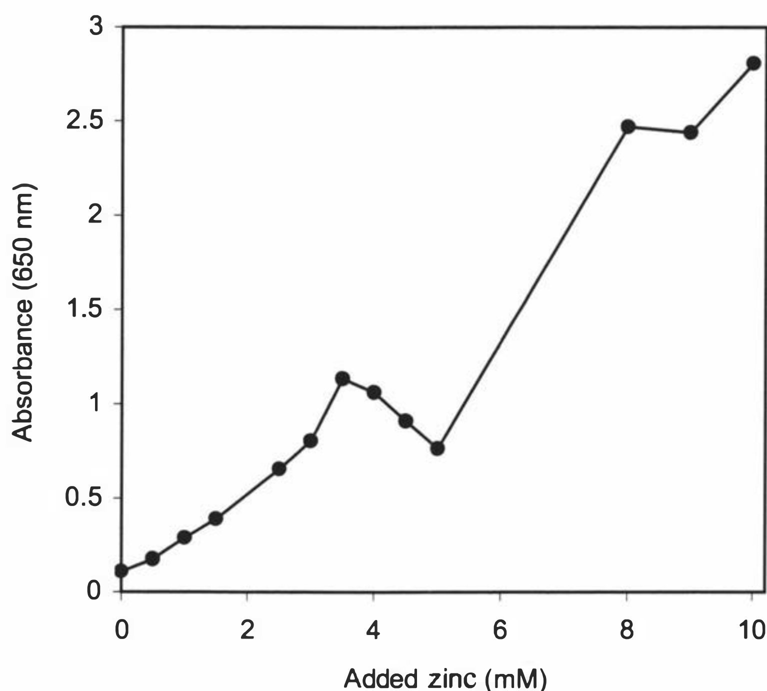


Figure 5.16. Turbidity (at 650 nm) of zinc sulphate–WPI mixtures as a function of added zinc concentration.

5.5.1. Solubility of Individual Whey Proteins in Zinc Sulphate–WPI Mixtures

The effect of the addition of zinc on the solubility of individual proteins in WPI was determined using SDS-PAGE, as described in Section 5.3.1.

The intensity of the α-La band was not affected by zinc addition up to approximately 4 mM (Figure 5.17), but decreased gradually at higher zinc concentrations (Figure 5.18); the α-La band was very faint at approximately 9 mM added zinc. The intensity of the β-Lg band was only slightly affected by the addition of zinc at up to 8 mM, but the β-Lg band appeared to decrease in intensity at higher zinc concentrations (Figure 5.18).

The decrease in the solubility of α-La at low added zinc concentrations could possibly explain the precipitation of zinc sulphate at above 3 mM added zinc. The added zinc may have been more associated with α-La rather than β-Lg at these concentrations.

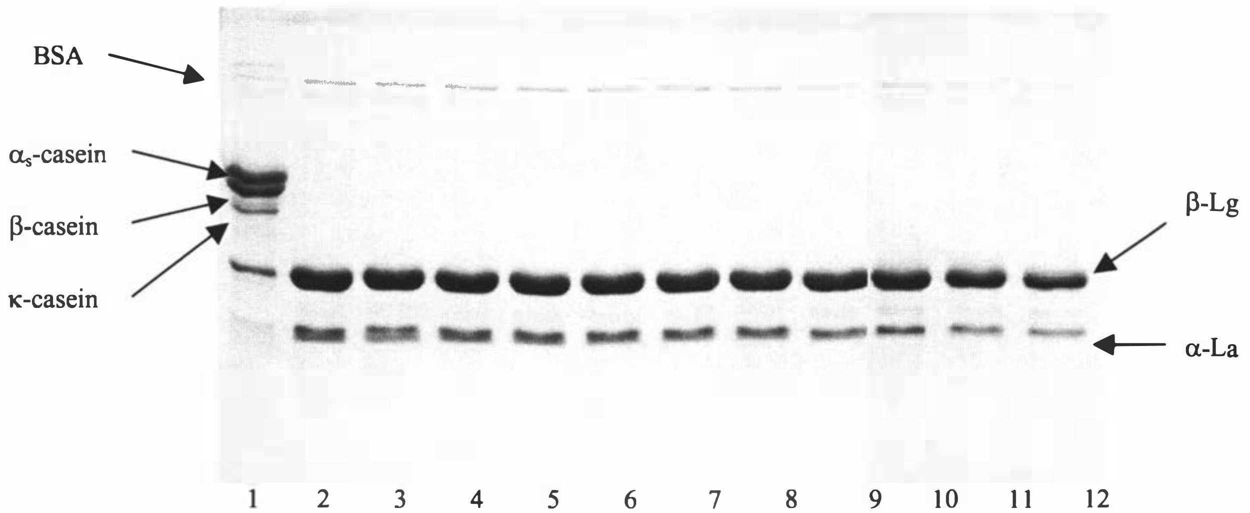


Figure 5.17. SDS-PAGE patterns of: skim milk (1), WPI solution without addition of zinc before centrifugation (2), the supernatant obtained after centrifugation (3), and zinc sulphate–WPI mixtures after centrifugation with added zinc at 2 mM (4), 3 mM (5), 4 mM (6), 5 mM (7), 6 mM (8), 7 mM (9), 8 mM (10), 9 mM (11) and 10 mM (12). Samples were dissolved in 50 mM HEPES buffer at pH 6.6.

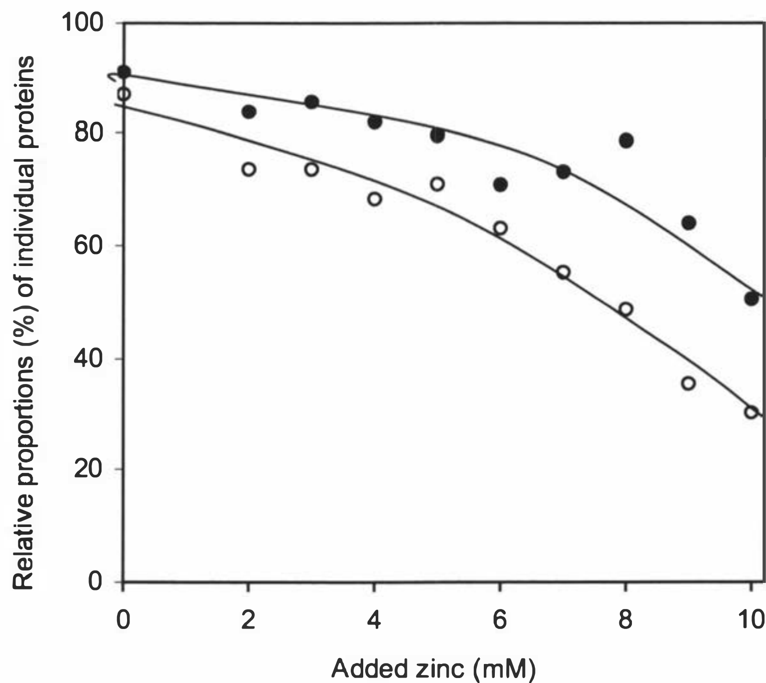


Figure 5.18. Proportions of individual proteins in the supernatant of WPI solutions: β-Lg (•) and α-La (o) as affected by the addition of zinc sulphate, in 50 mM HEPES buffer at pH 6.6.

5.6. Binding of Zinc to WPI

As mentioned in Section 5.5, addition of zinc at above a certain concentration to a WPI solution caused precipitation of the whey proteins and also of the added zinc. Hence, the zinc bound to WPI could be separated into two fractions: the zinc bound to soluble protein and the zinc bound to insoluble protein. The distribution of the total bound zinc between the soluble and insoluble fractions of WPI was calculated as described in Section 4.4 and the results are shown in Figure 5.19.

Approximately 90% of the zinc was bound to the soluble protein at up to 1.5 mM added zinc sulphate (Figure 5.19). At ≥ 2 mM added zinc, the relative proportions of zinc bound to soluble protein decreased with increasing added zinc concentration. This decrease was probably due to the precipitation of the zinc and the whey proteins from the zinc–WPI mixture (Figure 5.15).

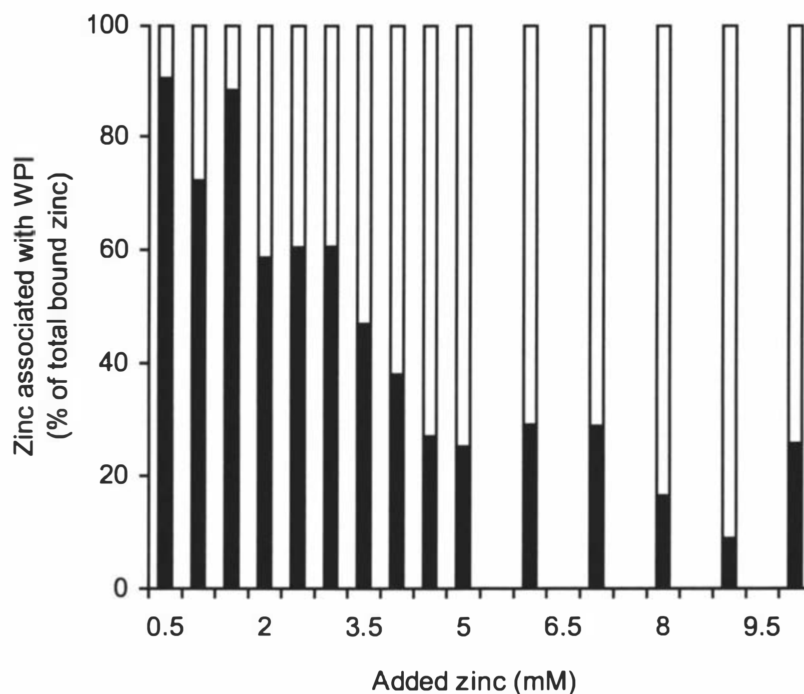


Figure 5.19. Distribution of bound zinc (as a percentage of the total bound zinc) between the soluble fraction (■) and the insoluble fraction (□) of WPI dissolved in 50 mM HEPES buffer at pH 6.6.

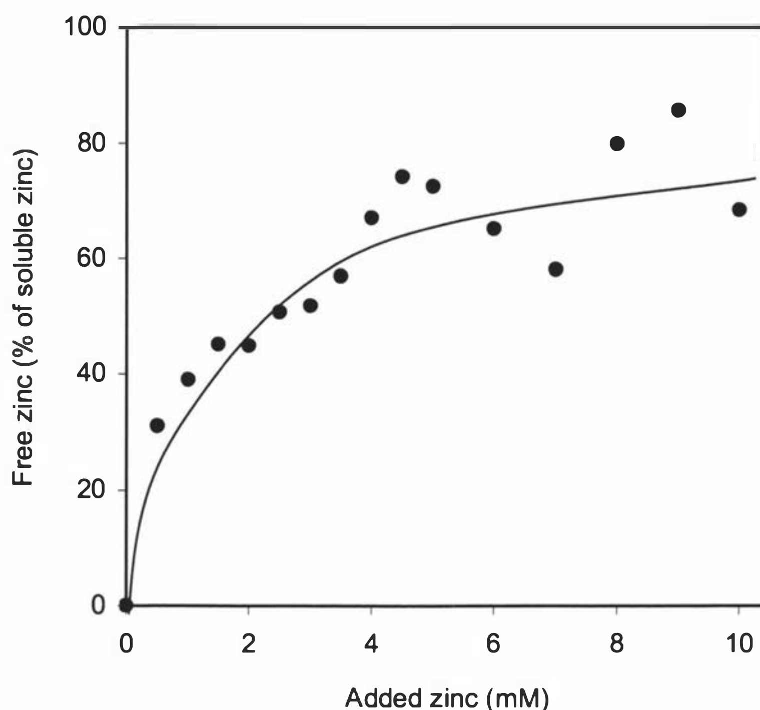


Figure 5.20. Amount of 'free' soluble zinc (i.e. in the ultrafiltration permeate) after ultrafiltration of zinc–WPI mixtures, in 50 mM HEPES buffer at pH 6.6.

5.6.1. Binding of Zinc to Whey Proteins in the Soluble Fraction

The binding of zinc to WPI in the soluble fraction was analysed using ultrafiltration. The amount of free zinc (zinc in the permeate) was plotted as a percentage of the amount of zinc that was in the supernatant (Figure 5.20). It appears that whey protein could bind only a relatively small amount of zinc, even at low concentrations of added zinc (0.5 mM). As expected, as the added zinc concentration increased, the proportion of free zinc increased.

Figure 5.21 shows the binding isotherm of zinc to WPI in terms of mol Zn bound/mol protein, using an MW of 18,000 Da for whey proteins. The binding of zinc to whey protein increased and levelled off at approximately 2 moles of zinc per mole of protein (approximately 7 mg Zn/g protein) before the whey proteins began to precipitate.

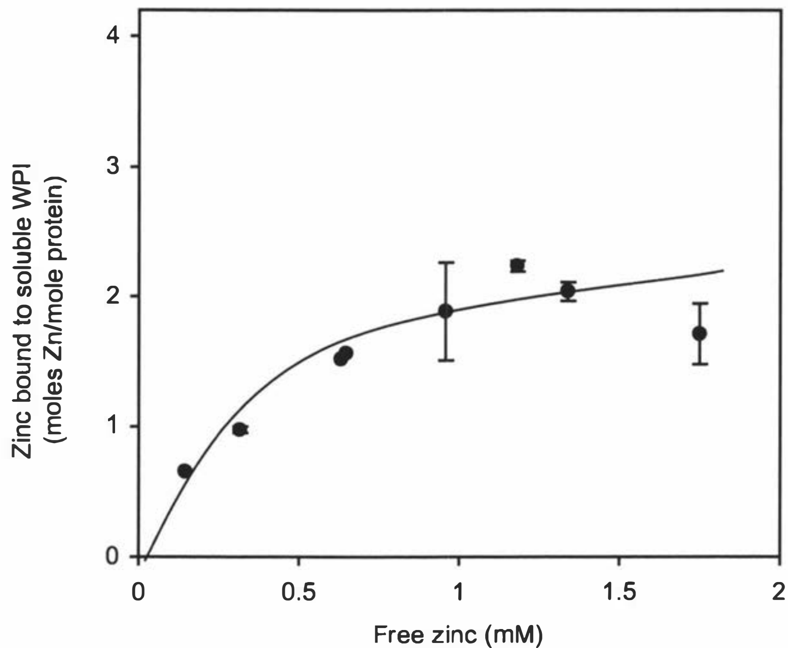


Figure 5.21. Binding of zinc to soluble WPI, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

5.6.2. Binding Sites and Binding Constants

The number of binding sites (n) and the apparent association constant (K_{app}) of the binding of zinc to WPI were evaluated using the Scatchard plot, as described in Section 4.4.3.

The Scatchard plot of the binding of zinc to WPI (Figure 5.22) could be fitted into a straight line at low molar ratios of zinc to whey protein. The Scatchard plot indicates that there were high affinity binding sites with specific binding. The number of high affinity binding sites (n) was calculated to be approximately 3.2 and the log K_{app} value was 3.19.

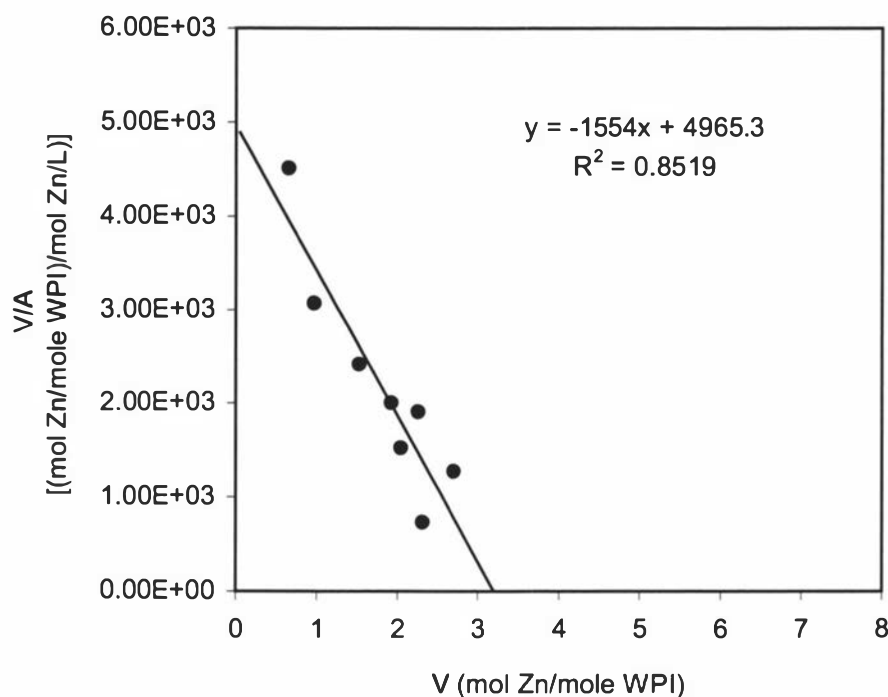


Figure 5.22. Scatchard plot for the binding of zinc to WPI, in 50 mM HEPES buffer at pH 6.6.

In this experiment, it was found that WPI had a lower ability to bind zinc (approximately 2 moles Zn/mole protein) (Figure 5.21) than did sodium caseinate (approximately 5 moles Zn/mole protein) (Figure 5.8). This is essentially in agreement with the findings of Cousins and Smith (1980), Harzer and Kauer (1982), Blakeborough *et al* (1983) and Singh *et al* (1989a). In terms of the individual proteins in WPI, Singh *et al* (1989a) showed that β-Lg, α-La and lactotransferrin had lower affinity than caseins for zinc but that BSA showed a zinc-binding capacity in excess of 8 atoms of zinc per mole of protein with approximately 5 high affinity sites. It is then likely that WPI has lower binding affinity for zinc than casein because the major proteins in the WPI used in the present study were β-Lg (70.4%) and α-La (24.3%) and not BSA (2.6%) (from SDS-PAGE results).

5.6.3. Effect of pH on the Binding of Zinc to WPI

The effect of pH on the binding of zinc to WPI was investigated at an added zinc concentration of 2 mM. At this concentration, there was no precipitation of zinc or protein.

Decreasing the pH of zinc–WPI mixtures from 7.0 to 4.0 did not change the solubility of the WPI. The solubility decreased slightly to approximately 80% at below pH 4.0 (Figure 5.23). Whey proteins have been reported to be soluble at low ionic strength over the entire pH range when they are in their native conformations (Mulvihill, 1992).

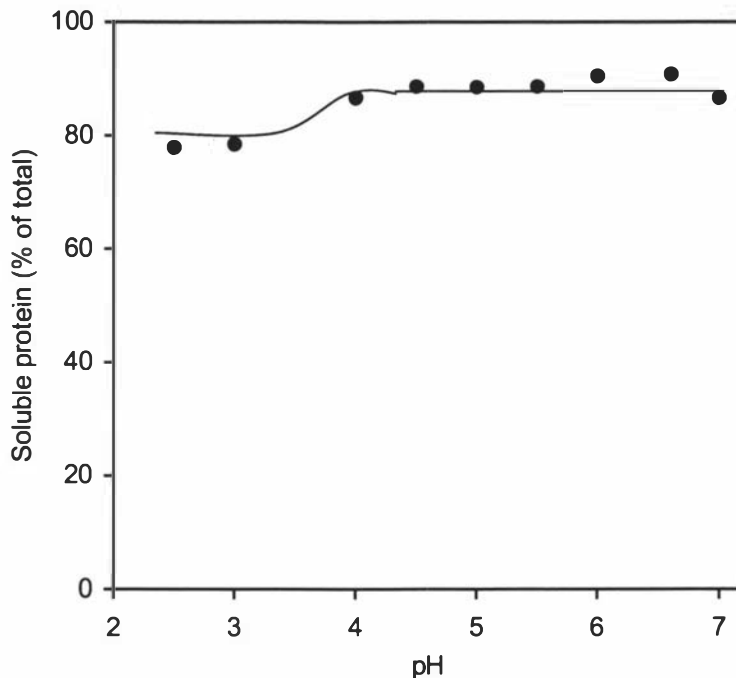


Figure 5.23. Effect of pH on the solubility of WPI in zinc sulphate–WPI mixtures containing 2 mM zinc, in 50 mM HEPES buffer.

Acidification of zinc–WPI mixtures markedly affected the ability of the whey proteins to bind zinc, as indicated by a large release of zinc into the ultrafiltration permeate. Approximately 30% of the added zinc was found in the permeate (i.e. free zinc) at pH 7.0. The release of zinc into the permeate increased almost linearly with a decrease in pH to 6.0; a further drop in the pH had a lesser effect (Figure 5.24).

Although the change in pH did not affect the solubility of the whey protein, it clearly changed the ability of WPI to bind zinc (Figure 5.25). The amount of zinc bound to WPI was very low at pH 2.5 but increased with increasing pH. The binding of zinc to WPI was found to be at a maximum at pH 7.0 (approximately 2 mol Zn/mol protein).

Little research has been carried out previously on the effects of pH on the binding ability of WPI. Baumy and Brule (1988a) studied the binding of zinc to α-La and β-Lg as affected by a change in the pH from 6.6 to 5.0. They found that the binding ability of both α-La and β-Lg decreased when the pH was decreased. The binding sites for divalent cations were reported to consist of a group of ionic amino acids. These amino acids, especially their carboxylic groups, are more or less dissociated with pH. Hence, when the pH was lowered, there was a decrease in ionisation and consequently a decrease in the zinc-binding ability.

The change in the structure of the individual whey proteins, i.e. α-La and β-Lg, during acidification might also cause a decrease in their ability to bind zinc. α-La undergoes a conformational change at around pH 4.0 and causes a release of Ca^{2+}, which is tightly bound at higher pH (de Wit, 1989). This conformational change of α-La might also cause the release of bound zinc at low pH. A conformational change of β-Lg from a stable dimer (at pH 6.7–5.2) to an octamer (at pH 5.2–3.5) or a monomer (below pH 3.5) might also contribute to the decrease in its ability to bind zinc (de Wit, 1989).

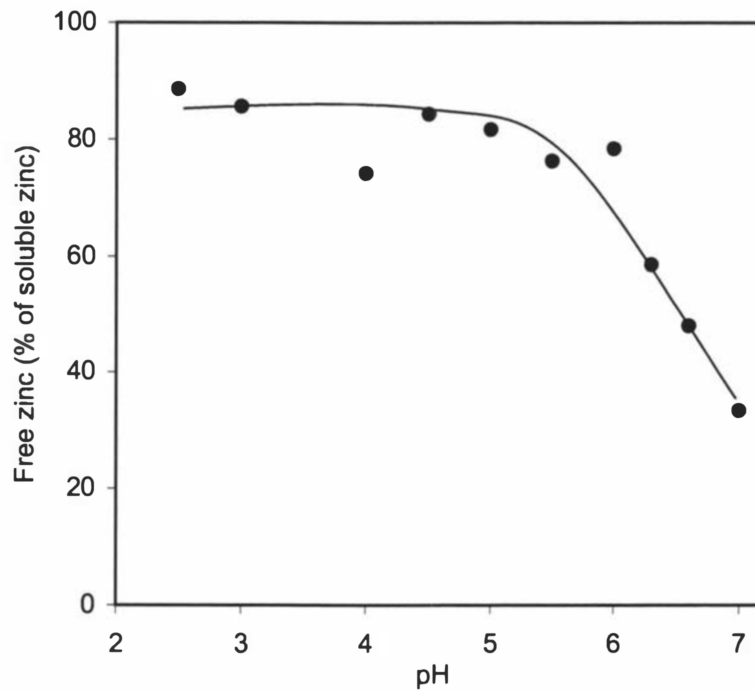


Figure 5.24. Effect of the pH of zinc–WPI mixtures (in 50 mM HEPES buffer) on the amount of free zinc in the ultrafiltration permeate (calculated as a percentage of the soluble zinc in the supernatant). The total concentration of zinc in the original solution was 2 mM.

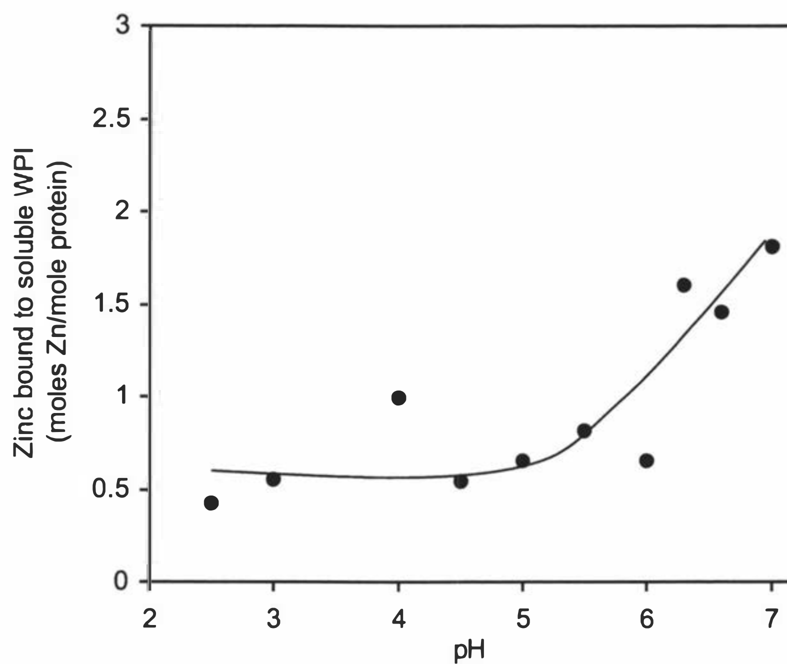


Figure 5.25. Amount of zinc bound to soluble WPI as affected by pH, in 50 mM HEPES buffer.

5.6.4. Effect of Ionic Strength on the Binding of Zinc to WPI

An increase in the NaCl concentration from 0.1 to 0.5 M of zinc–WPI solutions containing 2 mM added zinc sulphate caused no significant change in the solubility of WPI (Table 5.2). The free zinc concentration in the ultrafiltration permeate increased when the ionic strength was increased from 0.1 to 0.25 M and then decreased at 0.5 M NaCl (Table 5.2). As a result, the amount of zinc bound to soluble WPI was not affected by a change in the ionic strength from 0.1 to 0.25 M but the amount increased slightly when the ionic strength was changed from 0.25 to 0.5 M.

The results of Baumy and Brule (1988a) showed a decrease in the amount of zinc bound to α-La and β-Lg when the ionic strength was increased from 0 to 0.1 M (at pH 6.6). However, their results cannot be compared with the current experiments because of the different levels of ionic strength used. The ionic strength range investigated by Baumy and Brule (1988a) (0–0.1 M) was not covered in the present study.

Table 5.2. Effect of ionic strength on the ability of WPI to bind zinc in zinc–WPI mixtures (containing 2 mM zinc sulphate in 50 mM HEPES buffer at pH 6.6)

Ionic strength (M)	Soluble protein (% of total)	'Free' zinc (% of soluble zinc)	Soluble zinc bound to soluble WPI (mg Zn/g protein)
0.1	83.6	56.9	5.3
0.25	85.8	61.2	5.0
0.5	83.9	55.5	6.5

5.7. Solubility of Zinc and MPC in Zinc Sulphate–MPC Mixtures

The solubility of zinc in the presence of MPC was low; approximately 60% of the added zinc was soluble even at low concentrations of added zinc sulphate. This solubility decreased almost linearly up to approximately 3 mM added zinc and only approximately 30% of the added zinc was found to be soluble at above this concentration (Figure 5.26).

When the solubility of a 1% solution of MPC in the absence of zinc (0 mM added zinc) was investigated under the experimental conditions, it was found that only approximately 70% of the protein remained in the supernatant (Figure 5.27). As MPC comprises mostly casein micelles (Mulvihill, 1992), the decrease in MPC solubility in the absence of zinc reflects the sedimentation of large casein micelles upon centrifugation.

Addition of zinc at up to 2 mM had no effect on the solubility of the protein. Between 2 and 8 mM added zinc, the protein solubility decreased almost linearly, to approximately 20% at 8 mM added zinc (Figure 5.27).

The increase in the turbidity of zinc–MPC mixtures (Figure 5.28) as the added zinc concentration was increased indicated that aggregates were formed when zinc was added to the MPC solution. As expected, the turbidity of the supernatants decreased at high concentrations of added zinc.

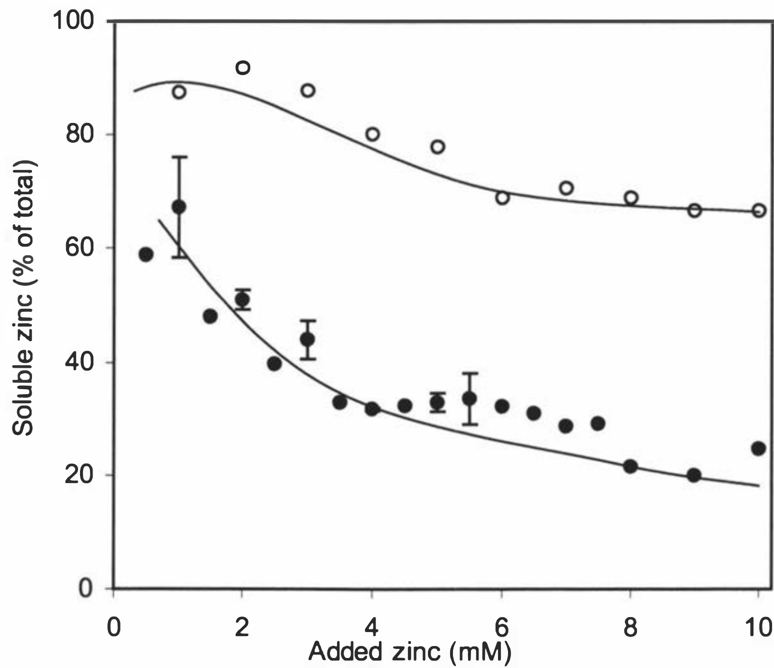


Figure 5.26. Solubility of zinc in the absence (o) and in the presence (•) of MPC, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

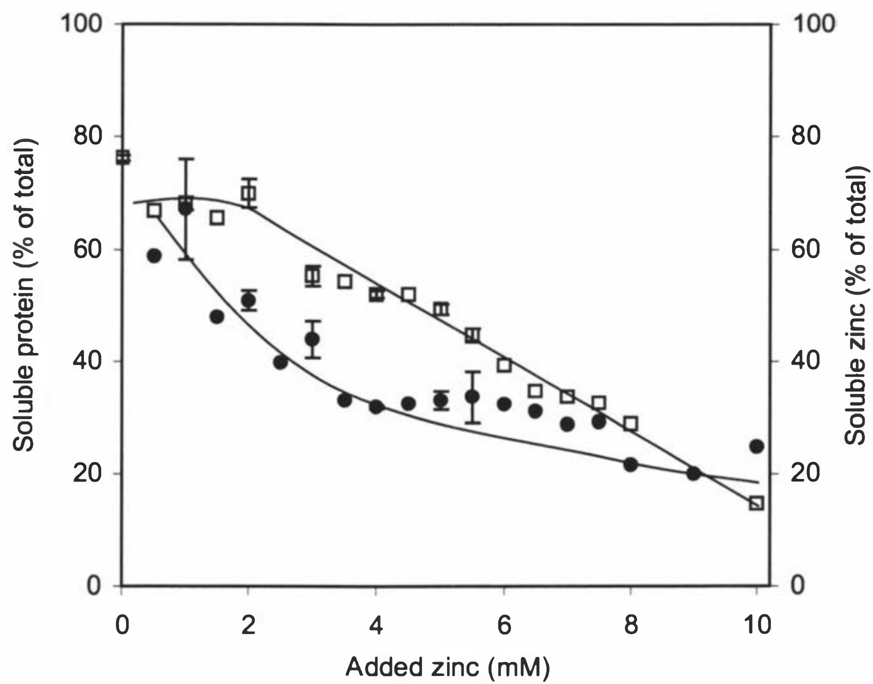


Figure 5.27. Solubility of the protein in MPC (□) and zinc (•) in zinc–MPC mixtures, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

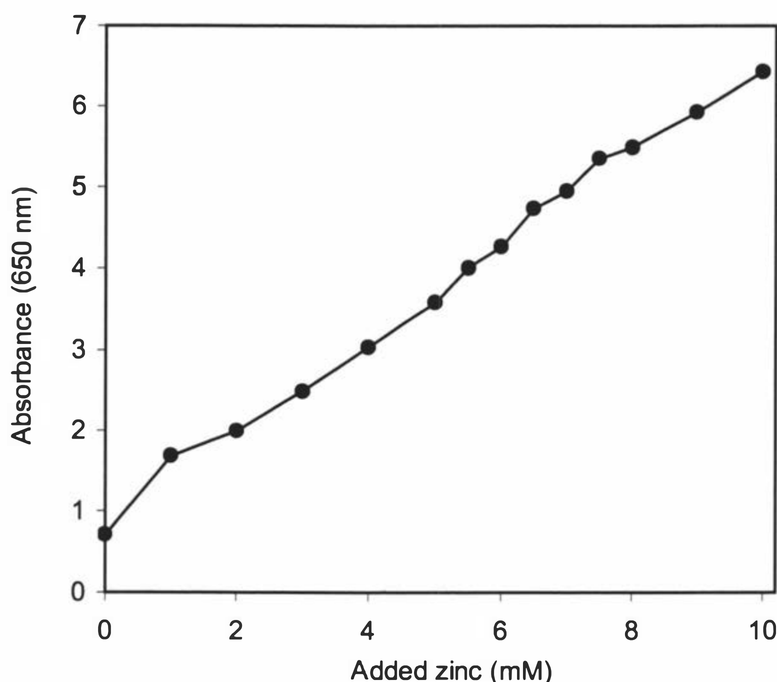


Figure 5.28. Turbidity (650 nm) of zinc–MPC mixtures at different added zinc concentrations.

When the solubilities of protein and zinc in the mixture were compared (Figure 5.27), it was found that considerable amounts of zinc sedimented at between 0 and 2 mM added zinc concentration, which may have been due to the association of the zinc ions with the large casein micelles in MPC because zinc sulphate in the absence of protein shows much higher solubility (Figure 5.26). These large casein micelles naturally sedimented under the centrifugation conditions used, causing a decrease in the solubility of the zinc.

It was reported that approximately 90% of the zinc in bovine skim milk was associated with casein micelles and sedimented when the micelles were separated using ultracentrifugation (Singh *et al*, 1989b). Similar results were found by Blakeborough *et al* (1983). Singh *et al* (1989b) found that casein micelles incorporated considerable amounts of zinc added to skim milk as zinc chloride. The fact that the amount of soluble protein in the MPC did not change between 0 and 2 mM added zinc, although the amount of soluble zinc had decreased, suggests that those large casein micelles

could probably bind more of the added zinc before their solubility decreased. The solubility of the protein in the MPC started to decrease at above 2 mM added zinc. This may have been due to further interactions of the added zinc ions with casein micelles in the MPC, causing aggregation and hence increased sedimentation.

5.7.1. Solubility of Individual Protein Fractions in Zinc Sulphate–MPC Mixtures

The solubilities of individual protein fractions in MPC were investigated. The mixtures containing 1% MPC and various levels of zinc sulphate were centrifuged and the supernatants were analysed using SDS-PAGE.

Figure 5.29 shows that MPC consists of casein fractions and whey protein fractions. The intensity of the casein bands (α_s-, β- and κ-caseins) was not greatly affected by addition of zinc sulphate at up to approximately 3 mM. The intensity of the individual casein bands decreased slowly at ≥ 3 mM added zinc, and was very low at above 8 mM added zinc. Among the three individual caseins, α_s-casein appeared to be more affected by the addition of zinc than β- and κ-caseins. The intensities of the whey protein fraction (β-Lg and α-La) bands were not greatly affected by increasing amounts of added zinc, except at very high concentrations of added zinc (9 and 10 mM).

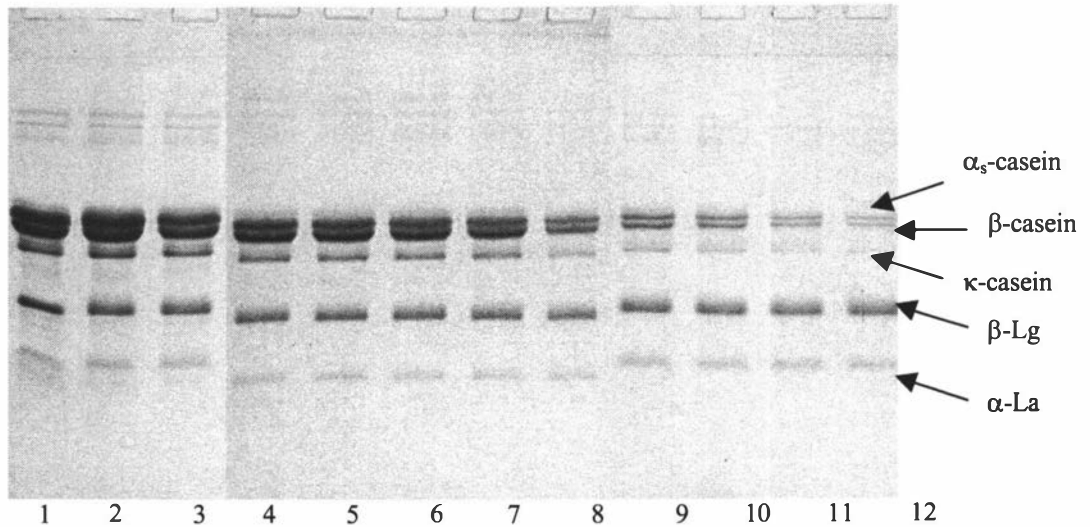


Figure 5.29. SDS-PAGE patterns of: skim milk (1), MPC solution without addition of zinc before centrifugation (2), the supernatant obtained after centrifugation (3), and zinc sulphate–MPC mixtures after centrifugation with added zinc at 1 mM (4), 2 mM (5), 3 mM (6), 4 mM (7), 6 mM (8), 7 mM (9), 8 mM (10), 9 mM (11) and 10 mM (12). Samples were dissolved in 50 mM HEPES buffer at pH 6.6.

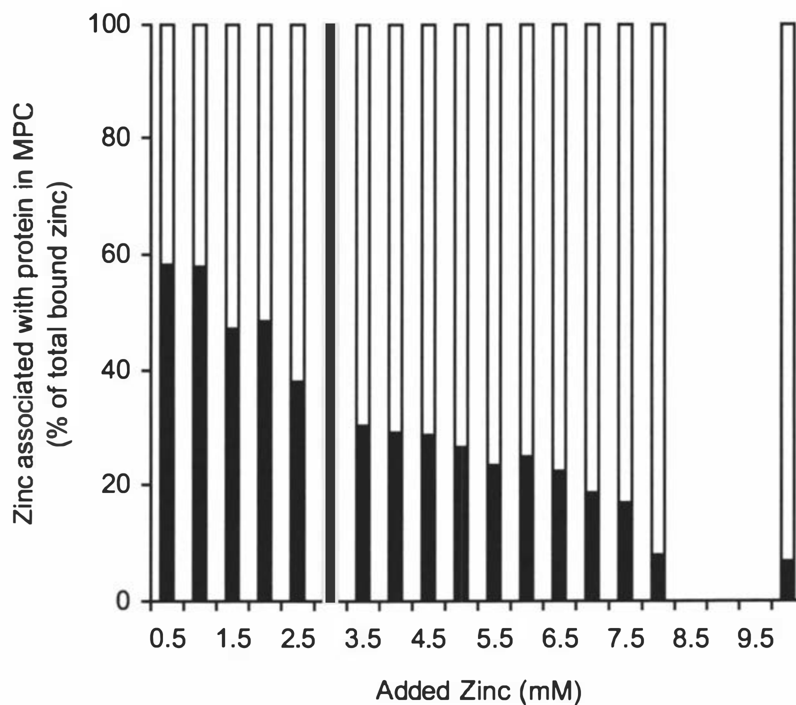


Figure 5.30. Distribution of bound zinc (as a percentage of the total bound zinc) between the soluble fraction (■) and the insoluble fraction (□) of MPC dissolved in 50 mM HEPES buffer at pH 6.6.

5.8. Binding of Zinc to MPC

The distribution of the total bound zinc between the soluble and insoluble fractions of MPC is shown in Figure 5.30.

With increasing amounts of added zinc, the relative proportions of zinc bound to soluble protein decreased gradually (Figure 5.30). At > 8 mM added zinc, less than 10% of the total zinc was bound to soluble protein and the remainder had sedimented along with the protein. The reason for the decreasing amount of soluble bound zinc was that the larger casein micelles (with bound zinc) in the MPC sedimented. At > 8 mM added zinc, the amount of caseins in the MPC had decreased and only the whey proteins remained soluble (Figure 5.29). As expected from the finding that the caseins have higher affinity than the whey proteins for zinc, the amount of soluble zinc associated with soluble protein was very low.

5.8.1. Binding of Zinc to MPC in the Soluble Fraction

As mentioned in Section 5.4.1, the binding of soluble zinc to MPC was investigated in more detail using ultrafiltration. The amount of free zinc in the ultrafiltration permeate as a function of added zinc is plotted in Figure 5.31. The binding isotherm of zinc to soluble MPC in terms of milligrams of zinc per gram of protein (the MW of MPC is not known) is plotted in Figure 5.32.

The amount of free zinc found in the ultrafiltration permeate was very low at up to 2 mM added zinc (Figure 5.31), indicating that all of the soluble zinc was bound to the soluble fraction of MPC. The binding profile showed a sharp increase in the amount of zinc bound to MPC (Figure 5.32) because of the small amount of free zinc.

The amount of free zinc began to increase at ≥ 2 mM added zinc (Figure 5.31). The increasing amount of free zinc at these concentrations may have been due to the fact that the proportion of soluble casein in the MPC had decreased at ≥ 2 mM added zinc

(Figure 5.29). Because caseins are known to have high affinity for binding to zinc, the decrease in the amount of soluble casein decreased the ability of MPC to bind zinc. Hence, more zinc ions were released as free zinc.

The binding of zinc to soluble MPC increased linearly as the concentration of added zinc increased and the binding isotherm levelled off at 25 mg Zn/g protein (Figure 5.32).

The binding affinity of MPC for zinc and its number of binding sites could not be determined using the Scatchard equation because the MW of MPC is not known.

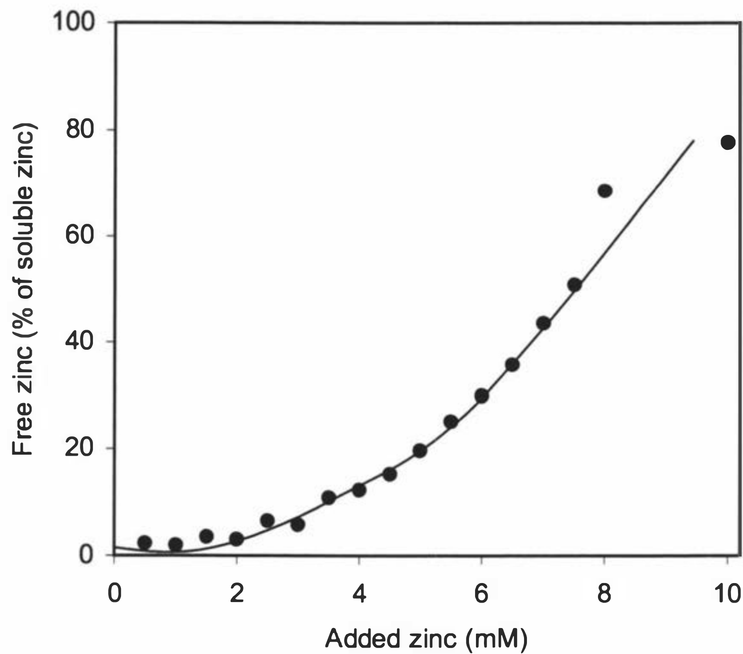


Figure 5.31. Amount of 'free' soluble zinc (i.e. in the ultrafiltration permeate) after ultrafiltration of zinc sulphate–MPC mixtures, in 50 mM HEPES buffer at pH 6.6.

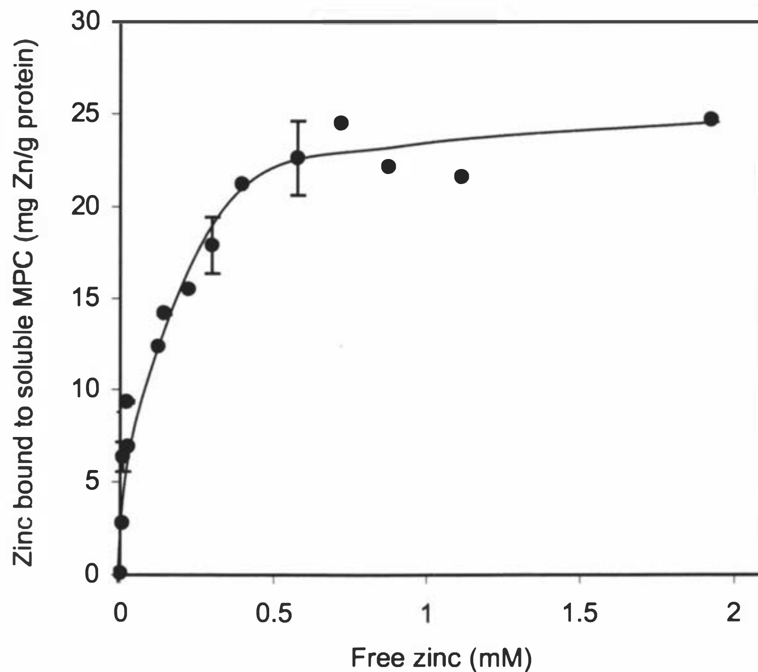


Figure 5.32. Binding of zinc to soluble MPC, in 50 mM HEPES buffer at pH 6.6. Bars indicate standard errors.

5.8.2. Binding of Zinc to MPC in the Insoluble Fraction

It was found that the binding of zinc caused precipitation of MPC and that the binding of zinc to the insoluble fraction of MPC increased with increasing amounts of added zinc sulphate (Figure 5.30). It was postulated that the added zinc was bound to the larger casein micelles in MPC, and that they then co-sedimented. However, the amount of zinc bound within the insoluble fraction of MPC could not be determined because the insoluble fraction was not quantified.

5.8.3. Effect of pH on the Binding of Zinc to MPC

The effect of pH on the binding of zinc to MPC was investigated at a zinc concentration of 2 mM. The procedure was carried out as described in Section 5.4.4.

Adjustment of the pH in the range from 2.5 to 7.0 affected the solubility of MPC in HEPES buffer before the addition of zinc sulphate (Figure 5.33). The solubility of MPC was approximately 80% at pH 6.6. It then decreased to 50% as the pH was changed to 5.0 and was very low (approximately 10%) at pH 2.5 and 3.0.

Addition of 2 mM zinc sulphate affected the solubility of MPC in the zinc–MPC mixtures in pH range 2.5–7.0, under the conditions used in the experiment (Figure 5.34). Between pH 5.5 and 7.0, the solubility of MPC was approximately 60%. At pH 6.6, the solubility of MPC before the addition of zinc sulphate was 80% (Figure 5.33). This indicated that the addition of 2 mM zinc at pH 6.6 caused a further decrease in the solubility of MPC. A decrease in the pH from 5.0 to 2.5 caused a marked decrease in the solubility of MPC (Figure 5.34); under these pH conditions, the solubility of MPC was only approximately 20%.

Acidification also affected the solubility of the added zinc (measured as the percentage of zinc that remained in the supernatant) in zinc–MPC mixtures (Figure 5.34). The solubility of zinc was 60% at pH 7.0 and increased slightly to 80% when the pH was decreased to 5.5. Approximately 50% of the added zinc was soluble at pH 5.0, but > 90% of the added zinc was soluble at pH 2.5 and 3.0. However, most of the protein had precipitated at pH 5.0. This trend was similar to that seen in zinc–sodium caseinate mixtures (Section 5.4.4). Therefore, it is also likely that MPC was able to bind the added zinc at pH 5.0, which then co-precipitated with the proteins on centrifugation. This did not occur at pH 3.0 and 2.5 because most of the added zinc was found to remain soluble when most of the MPC had precipitated.

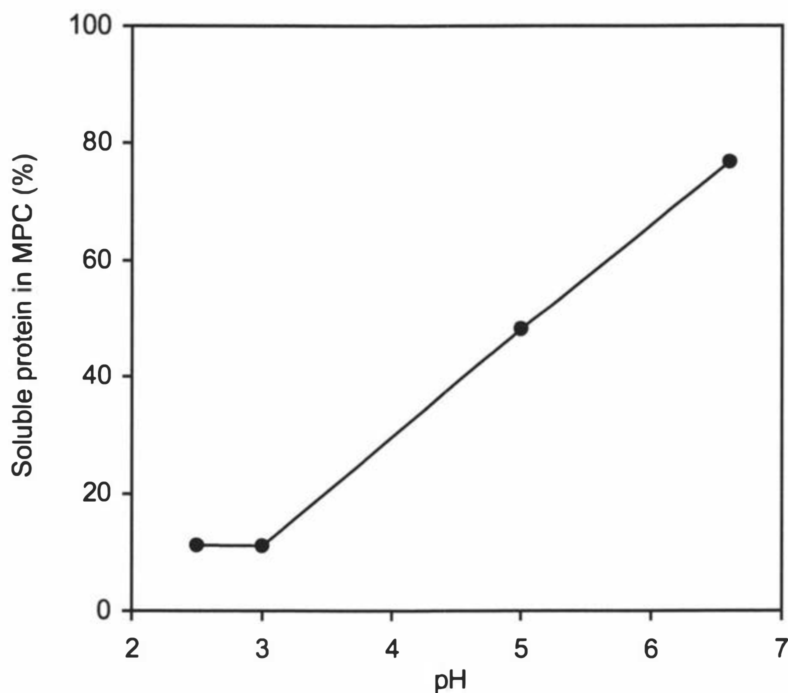


Figure 5.33. Effect of pH on the solubility of 1% MPC solution in 50 mM HEPES buffer *before* addition of 2 mM zinc sulphate.

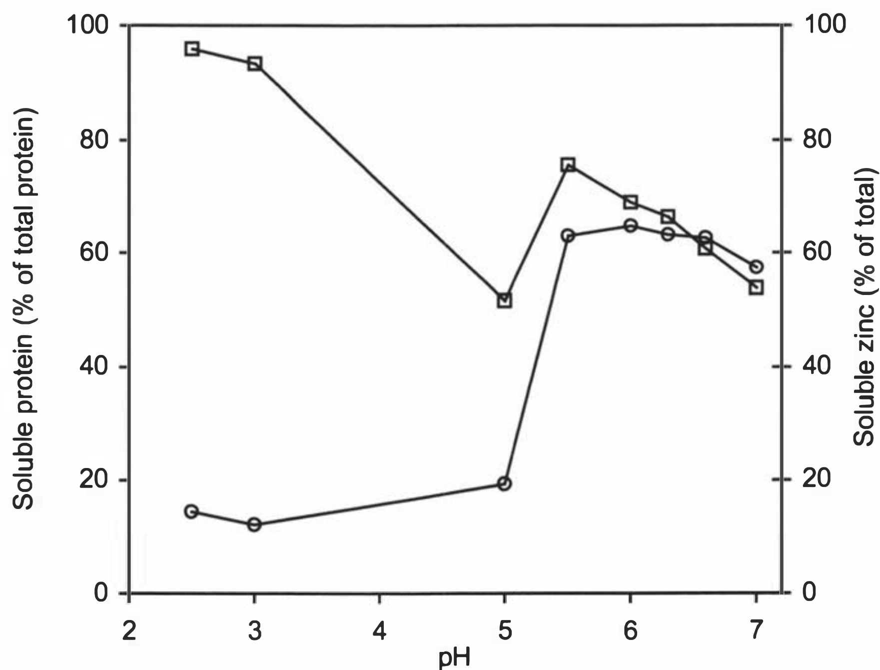


Figure 5.34. Effect of pH on the solubility of 1% MPC solution (o) and zinc sulphate (□) in zinc–MPC mixtures *after* addition of 2 mM zinc sulphate, in 50 mM HEPES buffer.

The ability of MPC to bind the added zinc was also affected by acidification. This can be seen from the amount of 'free' zinc that was released into the ultrafiltration permeate (Figure 5.35). When the pH was changed from 7.0 to 6.0, there was a slight increase in the amount of free zinc (approximately 10–20% increase) (Figure 5.35). There was a marked increase in the amount of free zinc at below pH 6.0, and almost all of the zinc was free at ≤ pH 5.0. This indicates that the binding of zinc to MPC was affected by a change in pH in the range from 2.5 to 7.0. Between pH 6.0 and 7.0, most of the MPC was still soluble and hence it was still able to bind most of the added zinc. However, at ≤ pH 5.0, the MPC had become insoluble and hence no soluble MPC was left to bind the added zinc.

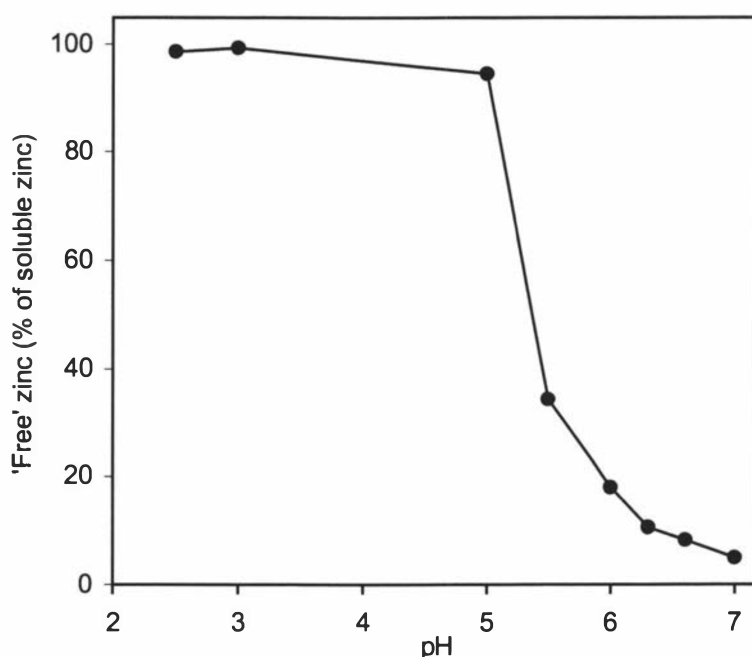


Figure 5.35. Effect of the pH of zinc–MPC mixtures (in 50 mM HEPES buffer) on the amount of free zinc in the ultrafiltration permeate (calculated as a percentage of the soluble zinc in the supernatant). The total zinc concentration in the original solution was 2 mM.

5.8.4. Effect of Ionic Strength on the Binding of Zinc to MPC

The effect of ionic strength (0.1, 0.25 and 0.5 M) on the binding of zinc to MPC was investigated in zinc–MPC mixtures containing 2 mM added zinc sulphate. At an ionic strength of 0.1 M, only 50% of the MPC was soluble (Table 5.3) but most of the soluble zinc was able to bind to the soluble MPC (only 10% of free zinc was found in the permeate). An increase in the ionic strength caused a slight increase in the amount of soluble MPC but did not cause much change in the amount of free zinc (Table 5.3).

The amount of soluble zinc bound to the soluble MPC was 8.3 mg Zn/g protein at 0.1 M ionic strength (Table 5.3). An increase in the ionic strength to 0.5 M caused a slight decrease in the amount of soluble zinc bound to the soluble MPC.

Table 5.3. Effect of ionic strength on the ability of MPC to bind zinc in zinc–MPC mixtures (containing 2 mM zinc sulphate in 50 mM HEPES buffer at pH 6.6)

Ionic strength (M)	Soluble MPC (% of total)	'Free' zinc (% of soluble zinc)	Soluble zinc bound to soluble MPC (mg Zn/g protein)
0.1	50.0	10.3	8.3
0.25	53.1	10.6	7.4
0.5	51.9	10.1	7.6

5.9. Discussion

Milk proteins are known to have the ability to bind polyvalent cations. Of the two major protein fractions in milk, the caseins possess better binding ability and bind cations (e.g. Ca^{2+}, Zn^{2+}) more strongly than the whey proteins (Fox and Mulvihill, 1990).

Caseinate is one of the main milk protein products and is a heterogeneous mixture consisting of α_{s1}-, α_{s2}-, β- and κ-caseins. These individual caseins have been reported to have the ability to bind zinc (Baumy and Brule, 1988b; Singh *et al*, 1989a). Because of the high content of phosphoserine residues, α_{s1}- and β-caseins bind Zn^{2+} strongly. α_{s1}-Casein, which has approximately 9 phosphoserine residues (Kinsella *et al*, 1989), has 11 binding sites for zinc (Singh *et al*, 1989a), whereas β-casein (approximately 5 phosphoserine residues) has 8 binding sites. κ-Casein, which contains only 1 phosphoserine residue has only 2 binding sites for Zn^{2+} (Singh *et al*, 1989a). The average association constant (log K_{app}) values were found to be similar (3.0–3.2), showing that the nature of the binding of zinc to the different caseins was similar.

The ability of β-casein to bind zinc has also been investigated by Baumy and Brule (1988b). It was reported that the amount of zinc bound to β-casein was 6.4 cations per mole of protein and that there were two kinds of zinc binding sites. The first 5 sites had higher affinity than the other 2 following sites. These sites were probably composed of 5 phosphoserines as the primary binding sites and 2 carboxylic sites. However, binding may also occur to aspartyl or glutamyl residues, as has been reported for the binding of Ca^{2+} to caseins (Fox and Mulvihill, 1990).

In the region where most of the caseinate was still soluble (at < 2.5 mM added zinc), zinc was bound to specific sites on the caseins and this binding occurred rapidly. Because of the binding ability of the individual casein fractions, it was possible that almost all of the binding sites in the α_s-, β- and κ-caseins in sodium caseinate were filled before they started to precipitate at a critical concentration of > 2.5 mM added zinc (Section 5.3). However, the binding ability of the individual casein fractions was not investigated in this mixed system.

It was found that the sodium caseinate used in the present study had approximately 6 binding sites for zinc. The binding sites for zinc in sodium caseinate are likely to be the same as the binding sites in the individual casein fractions, with the phosphoserine residues being the main binding sites (Cousins and Smith, 1980; Harzer and Kauer,

1982; Singh *et al*, 1989a; Gaucheron *et al*, 1997a). Other possible binding sites have also been mentioned, such as histidine and carboxylic groups, because dephosphorylation of the caseins markedly reduced the binding capacity but did not eliminate it (Singh *et al*, 1989a). The binding of zinc to caseins appears to involve the same binding sites as the binding of calcium. Dickson and Perkins (1971) found that the phosphoserine residue was the preferred binding site for calcium, with the carboxyl groups of aspartic acid and glutamic acid as the other possible binding sites.

In general, the binding of cations by the milk protein anions reduces the net charge on the protein and brings it closer to the isoelectric point (Kinsella, 1984), causing precipitation. Because the caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) are anionic proteins (Kinsella, 1984), they can aggregate on the addition of divalent cations, such as calcium, zinc, iron or copper (Harzer and Kauer, 1982; Kinsella, 1984; Baumy and Brule, 1988b; Fox and Mulvihill, 1990; Holt, 1992; Gaucheron *et al*, 1997a).

As mentioned before, precipitation of sodium caseinate occurred on the addition of > 2.5 mM zinc. It can be postulated that the precipitation of sodium caseinate caused by the addition of zinc occurs when all the sites are saturated (Baumy and Brule, 1988b). This precipitation corresponds to the negative charge neutralisation of the negative charges on the casein molecules by zinc, resulting in a decrease in the electrostatic repulsions between the casein molecules. This may enhance interactions between hydrophobic regions of the casein molecules. In addition, the binding of zinc to sodium caseinate, via the ionic linkage, could induce the formation of zinc ion bridges.

WPI is a purified whey protein product containing > 90% protein that consists of mainly β-Lg (70.4%), α-La (24.3%) and BSA (2.6%) (based on SDS-PAGE results). No data on the ability of WPI to bind zinc are available. However, Baumy and Brule (1988a) showed that both β-Lg and α-La have high affinity for zinc ions, with binding abilities of 2.5 and 4.5 ions of zinc respectively. Singh *et al* (1989a) reported that BSA, β-Lg and α-La showed at least two distinct classes of binding site, with log K_{app} values of 3.45, 4.0 and 3.0 respectively and a total number of binding sites of 22 for BSA and

approximately 2 for both β-Lg and α-La. Kronman (1989) provided evidence for the binding of zinc at two different sites, one a higher affinity site and the other a lower affinity site. Ren *et al* (1993) investigated the binding of zinc to human α-La using crystallography and found that zinc bound at a specific site in α-La with no significant secondary structural change.

The binding sites of α-La and β-Lg for divalent cations consist of a group of ionic amino acids such as carboxylic groups (Baumy and Brule, 1988a). The binding sites in β-Lg for Na^+, which have been reported to be carboxyls and imidazoles, are also other possible binding sites for zinc ions (Baker and Saroff, 1965).

In WPI, addition of zinc caused the binding of zinc to the whey protein fractions in WPI. It was found that WPI had approximately 3 binding sites for zinc, with a log K_{app} value of 3.19. The number of binding sites found in the present study is probably a combination of the binding sites in β-Lg and α-La (the major whey protein fractions in WPI).

The addition of > 4 mM zinc caused aggregation of a small proportion of the proteins in WPI. It seems that α-La was aggregated under these conditions and hence its solubility decreased. The solubility of β-Lg was not affected by zinc addition. It appears that the added zinc might be more associated with α-La rather than β-Lg. The precipitation of α-La as a result of the addition of zinc has been reported previously; precipitation of α-La occurred at zinc ion concentrations > 0.2 mM (Kronman, 1989; Ren *et al*, 1993). Kronman (1989) stated that the precipitation of α-La suggested that the protein underwent significant self-association via zinc binding. Ren *et al* (1993) stated that this precipitation might be due to the saturation of the binding of zinc to the sites in the proteins because α-La possesses several relatively strong Zn^{2+} binding sites, which were filled sequentially, the process being accompanied by protein aggregation.

For β-Lg, there is evidence that bovine β-Lg can bind both monovalent and divalent cations (Baumy and Brule, 1988a). However, precipitation has not been reported when binding occurs.

The binding ability of WPI was found to be lower than that of sodium caseinate. WPI had approximately 3 binding sites for zinc whereas sodium caseinate was found to have approximately 6 binding sites. The high binding affinity of sodium caseinate is not unexpected, because of its high casein content and hence its high level of phosphoserine residues. In contrast, WPI, which contains > 90% whey protein fractions and no caseins, contains no phosphoserine residue.

MPC is a milk protein product that contains both caseins and whey proteins with similar properties to those of their native forms in skim milk. The caseins in MPC are thus in the form of micelles bound together by colloidal calcium phosphate (CCP) (Munro, 2003). From SDS-PAGE, it was found that the MPC used in the present study comprised approximately 80% caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) and 20% whey proteins (mainly β-Lg and α-La).

In bovine milk, zinc is considered to be more associated (> 90% of the added zinc) with the casein fractions than the whey protein fractions (Harzer and Kauer, 1982; Blakeborough *et al*, 1983; Singh *et al*, 1989a, 1989b). There are two distinct micellar zinc fractions (Singh *et al*, 1989b); 32% of the zinc in skim milk is directly bound to the caseins and approximately 63% is associated with the CCP. It is likely that the addition of zinc to MPC would involve binding to both the casein micelles and the whey proteins. In the present study, it was possible that the added zinc (up to 2 mM) was associated, initially, with the larger casein micelles in MPC, which then co-sedimented upon centrifugation at 10,800 *g* for 20 min. This may have been due to a higher content of CCP in the large micelles. With increasing addition of zinc, the added zinc would have interacted with the smaller casein micelles. Increasing additions of zinc gradually led to aggregation and precipitation of the casein micelles. Because little casein remained soluble at high levels of zinc addition, the added zinc associated with

the whey protein fraction. Therefore, it can be postulated that the binding preference for zinc is to the casein fractions in MPC.

It was found that the soluble fraction of MPC could bind up to 25 mg Zn/g protein. This amount was greater than the amount of zinc bound to soluble sodium caseinate (approximately 14 mg Zn/g protein) and to soluble WPI (approximately 7 mg Zn/g protein). No published results on the binding of zinc to MPC are available, to compare with the present results. However, it is likely that MPC was able to bind more zinc because of the presence of CCP, which has been shown to bind zinc strongly (Singh *et al*, 1989b).

One of the factors that affected the binding ability of the milk protein products was the pH of the medium. In general, sodium caseinate solution is completely soluble at pH > 5.5 and minimum solubility is observed near its isoelectric point (around pH 4.0–4.5). Below pH 4.0, the solubility of sodium caseinate in the presence of NaCl is also at its minimum (Vojdani, 1996). The casein fractions in sodium caseinate are more sensitive to a change in pH and ionic strength than the colloidal-phosphate-containing casein micelles in milk (Kinsella, 1984). WPI is unique because it is soluble at low ionic strength over the entire pH range (Mulvihill, 1992).

The trend obtained in the present study showed that decreasing the pH (from pH 7.0 to 2.5) of the sodium caseinate and MPC solutions caused a decrease in the ability of the milk proteins to bind zinc. The decrease in binding was very pronounced at pH < 6.0.

In sodium caseinate, zinc is probably complexed to the casein molecules via the negatively charged phosphate groups. This decrease in the binding of zinc on reducing the pH may reflect the decrease in the ionisation state of the phosphoserine residues and also of histidine residues. This lowers the mineral-binding ability of the caseins because the phosphate residues are in the charge-free state. It indicates that Zn^{2+} is bound through ionic links (electrostatic) that are very sensitive to pH (Harzer and Kauer, 1982; Baumy and Brule, 1988b; Singh *et al*, 1989a; Gaucheron *et al*, 1997a).

The CCP in the casein micelles dissolves when the pH of milk is decreased (Pyne and McGann, 1960). This, combined with the change in the ionisation state of the phosphoserine residues, might reduce the ability of the protein to bind cations such as zinc. Therefore, when the pH of MPC was reduced (pH < 6.0), the zinc bound directly to the caseins and the zinc within the CCP decreased markedly.

Although decreasing the pH did not affect the solubility of the whey proteins, it affected the ability of the whey proteins to bind added zinc. Baumy and Brule (1988a) have also shown that reducing the pH (from 6.6 to 5) decreased the number of binding sites of zinc to β-Lg and α-La. The change in the ionisation state of negatively charged residues may have decreased the binding of zinc to WPI at low pH values. The change in the structure of the major individual whey proteins in WPI (i.e. α-La and β-Lg) during acidification might also cause a decrease in their ability to bind zinc. α-La undergoes a conformational change at around pH 4.0 and causes a release of Ca^{2+}, which is tightly bound at higher pH (de Wit, 1989). This conformational change of α-La might also cause the release of bound zinc at low pH. A conformational change of β-Lg from a stable dimer (at pH 6.7–5.2) to an octamer (at pH 5.2—3.5) or a monomer (below pH 3.5) might also contribute to the decrease in its ability to bind zinc (de Wit, 1989).

Addition of NaCl could affect the ability of the milk proteins to bind added cations through competition of the added sodium with the cation-binding sites in the milk protein molecules (Pyne, 1962). Changes in the ionic strength of the medium could also change the affinity of the binding sites (e.g. the phosphoserine residue in caseins), hence changing their ability to bind the added cations (Baumy and Brule, 1988b). In addition, the activity of the added cations might also change when the ionic strength is changed, which can affect the binding ability (Singh *et al*, 1988a).

In the present study, increasing the ionic strength from 0.1 to 0.5 M did not significantly change the ability of the milk proteins (sodium caseinate, WPI and MPC) to bind zinc. It has been reported that increasing the ionic strength caused a decrease in the binding

ability of individual casein fractions or individual whey protein fractions (Baumy and Brule, 1988a, 1988b; Singh *et al*, 1989a; Gaucheron *et al*, 1997a). It is possible that individual milk protein fractions are more sensitive to a change in the ionic strength of the medium than when they are present in a mixed system (e.g. sodium caseinate, WPI or MPC). In these mixed systems, the milk protein molecules might have better affinity for zinc. Hence, although there was an increase in the amount of sodium in the environment, there was no binding competition between added sodium and added zinc. The amount of added sodium might also be not significant enough to affect the ability of the milk proteins to bind zinc.

CHAPTER 6
EFFECT OF BINDING ON MINERAL–CATALYSED LIPID OXIDATION

6.1. Introduction

Fortification of foods with minerals is a common vehicle for delivering these minerals in required concentrations to the consumer. Many technological problems, however, occur when food products are fortified with minerals, due mainly to the minerals reacting with other components in the food system. One of such reaction is fat oxidation, which is known to be catalysed by transition metals, notably iron and copper.

Contamination of milk with iron is known to promote the development of a characteristic 'oxidised' flavour, as a result of lipid peroxidaton of milk fat (Hegenauer *et al*, 1979a). Consequently, dairy products have traditionally been protected from contamination with iron (Kurtz *et al*, 1973). However, when the iron is bound, it is unable to move between the ferrous and ferric states and hence its ability to catalyse oxidation is diminished (Hekmat and McMahon, 1998). In order to carry out fortification of dairy products with iron, researchers have therefore tried to complex the added iron or to use a chelated form of iron to minimise this oxidative deterioration (Carmichael *et al*, 1975; Hegenauer *et al*, 1979a).

An understanding of how metals catalyse lipid oxidation and cause organoleptic deterioration is of great importance. Therefore, in this experimental trial, the ability of iron– or zinc–protein complexes (which show good binding capacity) to influence the rate of lipid oxidation was investigated. The objectives of this experiment were:

- To investigate the extent of oxidation of linoleic acid induced by mineral-milk protein complexes (iron or zinc).

- To compare the pro-oxidant effect of the bound minerals with metals in their free form.

6.2. Oxidation Induced by Iron–Protein Mixtures

The extent of lipid oxidation caused by the mineral–protein mixtures was determined by incubating the complex with an emulsion containing linoleic acid and measuring selected oxidation products by the TBA test. Samples were held at 30°C in the dark, for up to 72 h. The oxidation activity of the metal ions bound to the mineral–protein mixtures was compared to the activity of the same metal ions in their free state (unbound) at the same concentration. The mineral concentration in the sample was 1 mM because at this concentration virtually all the added iron or zinc was bound to the milk proteins.

Linoleic acid with no additives was used as a control and was held under the same experimental conditions as the mineral–protein mixtures. In the three experimental trials (Figures 6.1, 6.2 and 6.3) the TBA reaction products from oxidised linoleic acid showed a relatively small increase in the absorbance readings at 532 nm over the incubation period; e.g. from 0.04 (0 h) to 0.38 (72 h) (Figure 6.3). These results suggest that the linoleic acid was not oxidised at the start of the experiment and was oxidised only slowly under the experimental conditions.

In a further set of experiments, linoleic acid held at 4°C in the dark was not oxidised over a 72-h period (absorbance readings at 0 and 72 h were 0.02 and 0.04 respectively). This suggests that linoleic acid is quite stable to oxidation at low temperatures over this time period.

Linoleic acid was oxidised at a faster rate when it was catalysed by free iron in the absence of proteins than by iron–protein mixtures (Figure 6.1, 6.2 and 6.3). The absorbance readings of the reaction products of oxidised linoleic acid catalysed by free iron and by iron–protein mixtures were approximately the same at the start of the

experimental trial. However, after 72 h, the absorbance readings of oxidised linoleic acid catalysed by free iron were approximately 6, 1.5 and 4 times higher than the corresponding absorbance readings for oxidised linoleic acid catalysed by iron–sodium caseinate, iron–WPI and iron–MPC mixtures, respectively. These results indicate that the ability of iron to catalyse lipid oxidation was reduced significantly when iron is bound (to protein) than when it is in its free form. Furthermore, the extent to which the different iron-protein mixtures reduced the rate of oxidation varied; the iron–WPI mixture seemed to show higher oxidation activity than either the iron–sodium caseinate mixture or the iron–MPC mixture.

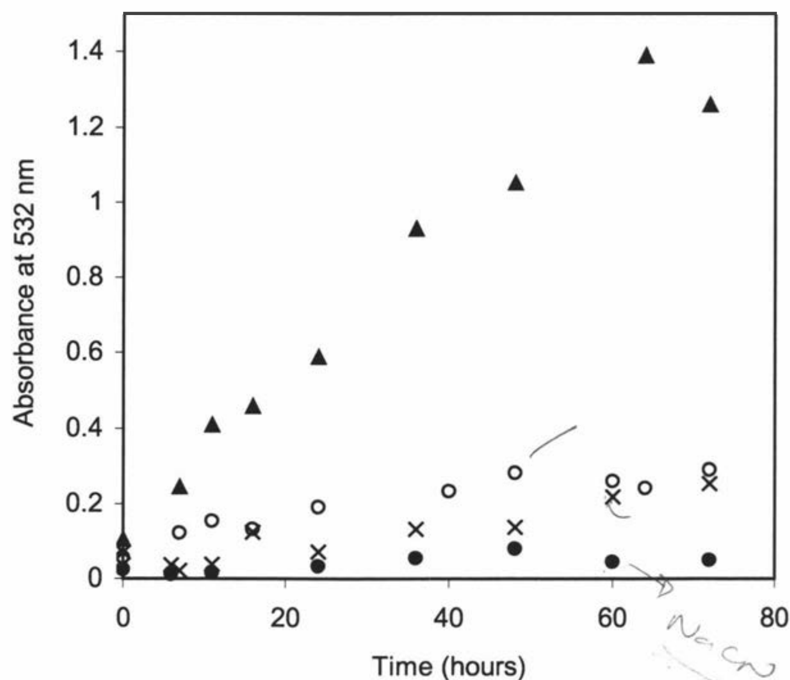


Figure 6.1. Absorbance readings of TBA reaction products from oxidised linoleic acid. Samples were held at 30°C, in the dark, for up to 72 h. Data are average of two trials. (**x**) Linoleic acid (control); (•) Linoleic acid with 1% sodium caseinate solution; (▲) Linoleic acid with free ferrous ions; (o) Linoleic acid with iron–sodium caseinate mixture.

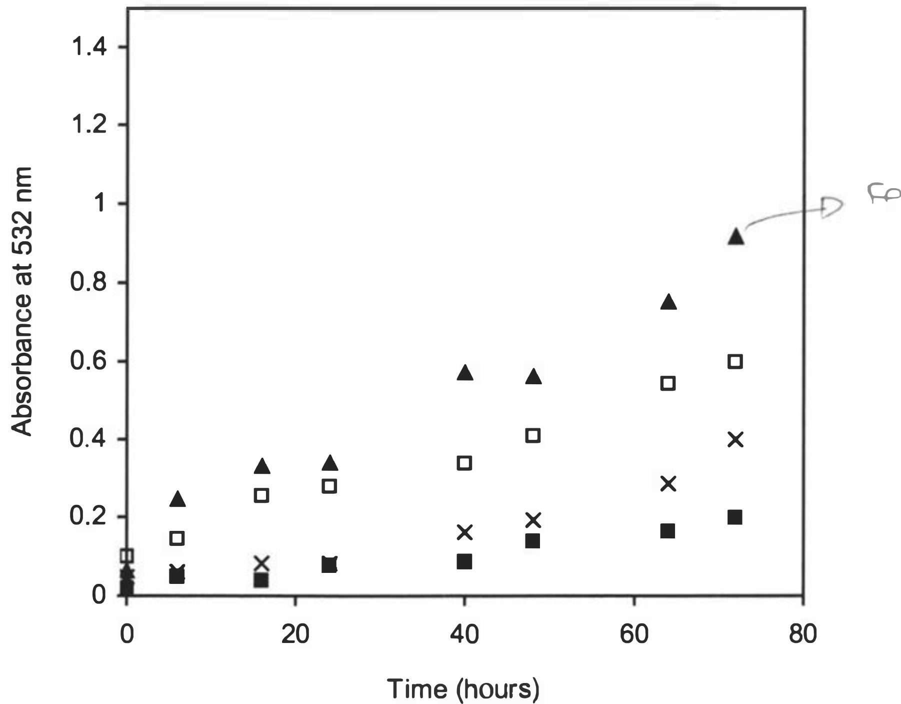


Figure 6.2. Absorbance readings of TBA reaction products from oxidised linoleic acid. Samples were held at 30°C, in the dark, for up to 72 h. Data are average of three trials. (**x**) Linoleic acid (control); (■) Linoleic acid with 1% WPI solution; (▲) Linoleic acid with free ferrous ions; (□) Linoleic acid with iron–WPI mixture.

Comparison between absorbance readings for the control (linoleic acid with no additives) and the sample containing linoleic acid and the iron–sodium caseinate mixture shows a similar degree of oxidation of linoleic acid (Figure 6.1). This suggests that under the experimental conditions of the trial, the iron–sodium caseinate mixture has a nil effect on the rate of oxidation of linoleic acid and does not appear to catalyse the reaction. The results for the iron–MPC mixture show a similar effect (Figure 6.3). However, in the case of WPI, the sample containing linoleic acid and the iron–WPI mixture oxidised at a faster rate than linoleic acid on its own (Figure 6.2).

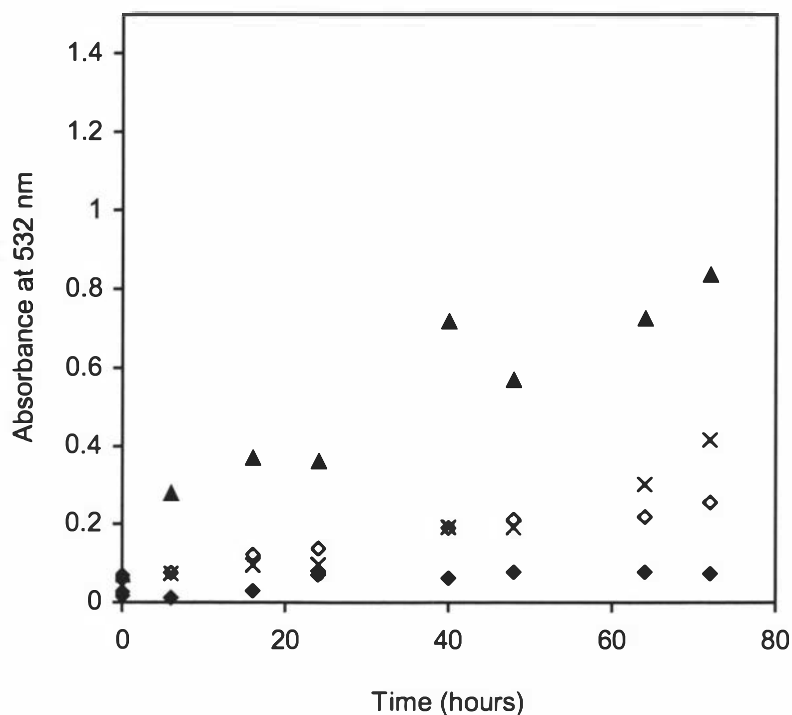


Figure 6.3. Absorbance readings of TBA reaction products from oxidised linoleic acid. Samples were held at 30°C, in the dark, for up to 72 h. Data are average of two trials. (**x**) Linoleic acid (control); (◆) Linoleic acid with 1% MPC solution; (▲) Linoleic acid with free ferrous ions; (◇) Linoleic acid with iron–MPC mixture.

Linoleic acid with no additives (control) was oxidised at a faster rate than linoleic acid in milk protein solutions (Figures 6.1, 6.2 and 6.3). Each of the milk protein solutions appeared to act as an anti-oxidant with the effect being greater for the sodium caseinate and MPC solutions than for the WPI solution. Indeed, for the linoleic acid–sodium caseinate solution, absorbance readings showed little increase over the incubation period (from 0.02 to 0.06) (Figure 6.1) suggesting that sodium caseinate had effectively prevented the oxidation of linoleic acid.

6.3. Oxidation Induced by Zinc–Protein Mixtures

Two experiment trials were carried out to determine the effect of the zinc–sodium caseinate mixture on the rate of oxidation of linoleic acid. In Figure 6.4 the absorbance readings of the TBA reaction products from oxidised linoleic acid for the different samples are plotted against time.

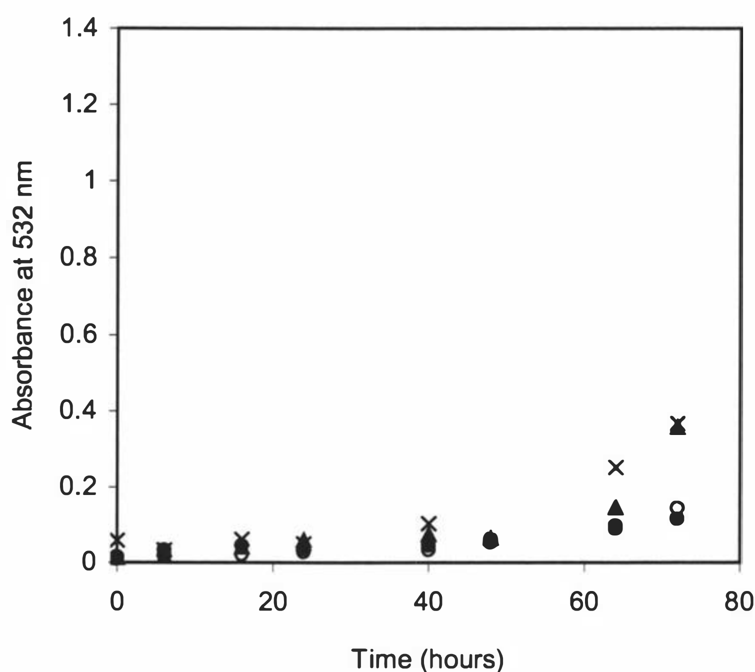


Figure 6.4. Absorbance readings of TBA reaction products from oxidised linoleic acid. Samples were held at 30°C, in the dark, for up to 72 h. Data are average of two trials. (**x**) Linoleic acid (control); (•) Linoleic acid with 1% sodium caseinate solution; (▲) Linoleic acid with free zinc ions; (**o**) Linoleic acid with zinc–sodium caseinate mixture.

Comparison between absorbance readings for the control (linoleic acid with no additives) and the sample containing linoleic acid and free zinc shows a similar degree of oxidation. Furthermore, the sample containing linoleic acid and the zinc–sodium caseinate mixture was oxidised to about the same extent as the sample containing

linoleic acid and sodium caseinate. Both these later samples showed slightly less oxidation than the control after 72 h.

These data show that free zinc does not appear to catalyse the oxidation of linoleic acid and that the presence of sodium caseinate may slow down the rate of oxidation to a small extent.

6.4. Discussion

The transition metals which possess two or more valency states with a suitable oxidation–reduction potential between them (e.g. copper and iron) are capable of undergoing reversible one–electron reactions. These metal ions are considered to act as pro-oxidants primarily by catalysing the decomposition of hydroperoxides (ROOH) as shown in the following equations (Labuza, 1971).

$$M^{n+} + ROOH \longrightarrow M^{(n+1)+} + OH^- + RO^\bullet \quad (1)$$

$$M^{(n+1)+} + ROOH \longrightarrow M^{n+} + H^+ + ROO^\bullet \quad (2)$$

Thus, small quantities of an appropriate metal ion can generate large numbers of reaction chains by cycling between the oxidised and reduced forms. Although iron and copper in their reduced states are effective reducers of ROOH (equation 1), they are less efficient oxidisers in their higher oxidation states (equation 2) (Labuza, 1971; O'Connor and O'Brien, 1995).

Furthermore, these metals may act as pro-oxidants by generating the active oxygen species, singlet oxygen, which can react directly with unsaturated fatty acids to produce hydroperoxides (Richardson and Korycka-Dahl, 1983).

The marked effect on the oxidation rate caused by the presence of free (unbound) iron is shown in the results in the present study (e.g. Figure 6.1). The rate of oxidation of

linoleic acid in the presence of free ferrous ions was found to be markedly higher than the control (linoleic acid with no additives).

The marked pro-oxidant effect of ferrous ions is in agreement with other published studies. Edmonson *et al* (1971) showed that enrichment of whole milk before pasteurisation with ferrous ions gave rise to oxidised flavours. In the review by Richardson and Korycka-Dahl (1983), a comprehensive study of the oxidation of linoleic acid showed that the effectiveness of metals at decomposing linoleic acid hydroperoxide was in the order $Fe^{2+} > Fe^{3+} > Cu^{2+}$. The Fe^{2+}-catalysed decomposition was about ten-fold faster than that with Fe^{3+}. A further study by Yen *et al* (1999) showed that among the pro-oxidants used in the trial (i.e. Fe^{2+}/H_2O_2, Fe^{3+}/Ascorbic acid or Fe^{3+}/H_2O_2/Ascorbic acid), Fe^{2+} and Fe^{3+} exhibited more stimulatory effects on the peroxidation of linoleic acid than the other pro-oxidants. They reported that adding Fe^{2+} to an unsaturated lipid would produce active oxygen species which then initiate the first step of the chain reaction of lipid peroxidation.

The present study showed that the rate of oxidation of linoleic acid was less when the iron is complexed to milk proteins, than when the iron is present in the free (unbound) form, regardless of the kinds of milk proteins (i.e. sodium caseinate, WPI or MPC) which bind the iron.

An investigation by Hegenauer *et al* (1979a) showed that chelated forms of iron are more preferable as supplements for milk than simple inorganic salts (ferrous sulphate or ferrous chloride). This is because the unbound ferrous ion could participate in the cyclic oxidation-reduction reactions, therefore amplifying its potential for catalysing lipid oxidation. In contrast, the chelated forms of iron, such as ferric-NTA, removed the metal from the environment of the lipid fraction which decreased the rate of oxidation. Therefore, for the same concentration, iron presented as ferric chelate (ferric lactobionate and ferric-NTA) caused less oxidation than free ferrous iron. This confirmed the study by Carmichael *et al* (1975). In addition, Wang and King (1973) stated that the use of ferric ammonium citrate to fortify milk could reduce the rate of

lipid oxidation induced by iron compared to ferrous sulphate. The ferric ammonium citrate was largely undissociated or was readily complexed to some component in milk thus inhibiting its lipid oxidative activity.

It is interesting to note that, in contrast to iron, copper in the presence of milk proteins, may enhance the rate of lipid oxidation compared to copper alone (Tappel, 1955). It has been postulated that the combination of a copper-protein complex with the phospholipids of the milk fat globule membrane is an important factor in the development of oxidised flavour in liquid milk (Samuelsson, 1966).

These two contrasting trends indicate that the effectiveness with which copper and iron act as pro-oxidants in milk may depend on the different interactions of the two metals with other milk constituents (e.g. milk proteins, phospholipids, ascorbic acids and thiols) (O'Connor and O'Brien, 1995).

In the current investigation, when milk proteins were present in the sample, the oxidation of linoleic acid was suppressed compared to the control (linoleic acid with no additives). Among the three milk proteins used, it was found that WPI was less effective than casein proteins (i.e. sodium caseinate and MPC) at reducing oxidation. This result is in agreement with some published studies. Taylor and Richardson (1980) found that the caseins had much greater antioxidant activity than the whey proteins. Reviews by Richardson and Korycka-Dahl (1983) and O'Connor and O'Brien (1995) also state that casein proteins have anti-oxidative properties, which appears to be related to their hydrophobic nature and the orientation of potential antioxidant side-chains of constituent amino acids at the lipid interface (Allen and Wrieden, 1982; Ericksson, 1982; Yee and Shipe, 1982). However, the antioxidative effect varies depending on the concentration of the amino acid itself, pH, temperature and concentration of trace metals (Labuza, 1971; Eriksson, 1982). For example, although caseins have little or no sulphydryl groups that are reported to provide antioxidant properties, they have significant antioxidant activity compared to β-Lg, which has almost all the sulphydryl groups of milk (Taylor and Richardson, 1980). Furthermore, linoleic acid oxidation

was shown to be inhibited by cysteine, histidine and alanine at pH 9.5, while at pH 7.5, cysteine was strongly pro-oxidative. Both histidine and alanine were antioxidative at low concentrations but pro-oxidative at higher concentrations (Eriksson, 1982).

Caseins may also act as antioxidants because they have the ability to bind pro-oxidant metals to their phosphoseryl residues (Manson and Cannon, 1978; Hegenauer *et al*, 1979a) which inhibits the catalytic effect of the metals (Allen and Wrieden, 1982). The present study has shown that iron added as ferrous sulphate was bound completely to the casein molecules in the sodium caseinate up to 4 mM concentration of added iron (Section 4.4). The soluble caseins and the casein micelles in MPC have also been shown to have the ability to bind significant amounts of iron (Section 4.8). Therefore, when the added iron was bound completely to casein molecules, the catalytic effect of iron can be prevented which leads to much lower rates of lipid oxidation.

Whey proteins are reported to be less effective as antioxidants than caseins (O'Connor and O'Brien, 1995). This is possibly due to the lower iron-binding ability of whey proteins. The present study found that for sodium caseinate and MPC, all iron was completely bound at relatively low concentrations of added iron. In other words, there was no free iron present (Figure 4.13 and 4.38). However, in the case of WPI, there was always a small amount of free iron even at low concentration of added iron (Figure 4.26). Thus, in a powder containing whey proteins (e.g. WPI), the free iron will be able to move between its different oxidation states, causing oxidation.

In the present study, the presence of free zinc did not alter the rate of oxidation of linoleic acid (Figure 6.4). This was the expected result because zinc has only one oxidation state and is not capable of undergoing reversible one-electron reductions. Hence, zinc is not able to catalyse the decomposition of hydroperoxides. To our knowledge, there are no published studies which show that zinc enhances the oxidation of fat.

CHAPTER 7
CONCLUDING DISCUSSION AND RECOMMENDATIONS

7.1. Concluding Remarks

The present study focused on determining the ability of different milk protein products (sodium caseinate, MPC and WPI) to bind iron and zinc. The binding properties of these milk protein products, in terms of binding isotherms, binding constants and number of binding sites, were characterised. The effect of solution conditions, such as pH and ionic strength, were also investigated. In addition, selected mineral–protein complexes were tested for their effect on lipid oxidation. The binding of iron and zinc to each milk protein product has been discussed in detail in the previous chapters. This chapter focuses on the similarities and differences in the behaviour of iron and zinc in binding to each milk protein product. In addition, the chapter identifies the areas where further work will be useful and how the information generated from this work could be used by the dairy industry to manufacture new protein ingredients.

For the binding study, the methodology used in the present study involved preparing mineral–protein mixtures by adding various quantities of the cations to a 1% protein solution at pH 6.6. Because iron and zinc behaved differently in aqueous solutions, the solubility of these metal ions was investigated initially. It was found that the solubility of ferrous sulphate was low under the conditions used in the present study (Figure 4.1), which was due largely to the formation of ferric hydroxides at the relatively high pH (6.6); the solubility of iron increased with increase in the concentration of ferrous sulphate. In contrast, zinc sulphate was mostly soluble, when dissolved in an aqueous solution and its solubility slightly decreased with concentration (Figure 5.1). This decrease was probably due to the formation of zinc hydroxide, which precipitated from solutions of salt by addition of bases. The different behaviour of the two metal ions could be due to the fact that iron is one of the transition metal ions that have more than

one valence state (Labuza, 1971) hence enabling it to form ferric hydroxides. These ferric hydroxides precipitate at a faster rate at high pH (> pH 5.5) (Eyerman *et al*, 1987) compared with zinc hydroxides.

In the presence of sodium caseinate (at pH 6.6), the solubility of ferrous sulphate improved markedly (Figure 4.5). Up to 4 mM of added iron, virtually all the added iron was bound to the protein molecules. Consequently, there was no free iron left in solution to react with hydroxides to form insoluble ferric hydroxide. This suggests that the solubility of ferrous sulphate can be improved markedly through its binding with caseinate molecules. This may have potential applications in iron fortification of nutritional beverages and drinks.

Iron and zinc binding properties of sodium caseinate, whey protein isolate (WPI) and milk protein concentrate (MPC) are summarised in Table 7.1. The binding properties of sodium caseinate for binding to zinc were qualitatively similar to that for binding to iron. Addition of zinc up to 2.5 mM resulted in binding of almost all the zinc to the casein molecules in sodium caseinate. It is likely that both iron and zinc bind to the same sites in casein molecules, which are mainly the clustered phosphoserine residues in caseins. Other possible weak binding sites such as carboxylic groups, phenolic groups, sulphydryl groups and imidazole groups may also be involved (Gaucheron *et al*, 1996, 1997a).

Addition of iron or zinc, above a certain critical concentration, induced the formation of casein aggregates which led to the loss of protein solubility. However, the critical concentrations were different for iron and zinc (Figure 4.5 and 5.3). In the case of zinc, > 2.5 mM of added zinc was required to cause the loss of solubility of caseins whereas above 4 mM iron was required to initiate protein aggregation. This aggregation and precipitation probably occurred when all the binding sites were saturated and could be attributed mainly to charge neutralisation by the added iron, causing a decrease in the electrostatic repulsions between the protein molecules. The differences between the effects of iron and zinc on protein aggregation may have been due to the different size

of the ions. It has been reported that if the number of binding sites for the metal ion and their relative distribution and space remain the same, then within a like-charged series, the smaller the ion, the more stable the complex (Gillard and Laurie, 1988). The stabilities of the complexes of the bivalent ions of the first transition series, irrespective of the particular ligand involved, usually vary in the Irving-Williams order: $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$ (Greenwood and Earnshaw, 1997). Therefore, it could be postulated that the iron could form a more stable complexes than zinc and thus more ferrous ions would be needed to precipitate the caseins.

It was found that the binding ability of casein molecules in sodium caseinate was higher for iron than that for zinc. Caseins were able to bind approximately 9 moles of iron per mole of protein in the soluble region while only 5 moles of zinc per mole protein was bound to caseins (Table 7.1). The number of binding sites for iron (n=14), calculated from the Scatchard plot, seems to be rather high and is probably not accurate. This could be due to problems associated with protein precipitation above a critical concentration of added iron. The average association constant for iron (Log K_{app} = 5.3) was higher than for zinc (Log K_{app} = 4.84). This is consistent with the findings of Baumy and Brule (1988b) for binding of different cations to β-casein, which follows the order: $Ca^{2+} < Mn^{2+} < Zn^{2+} < Cu^{2+} < Fe^{2+}$. The differences in the affinity of casein proteins for iron and zinc might be caused by the difference in the ionic radius and electronegativity of the cations. Electronegativity is defined as "the power of an atom *in a molecule* to attract electrons to itself" (Greenwood and Earnshaw, 1997). Iron has been reported to have a high electronegativity while the electronegativity for zinc is medium, thus it is likely that caseins have more binding preference for iron than zinc. However, the variation in the electronegativity across the transition metals are relatively small (Mingos, 1998).

In contrast to sodium caseinate, addition of iron to WPI did not significantly affect the solubility of the whey proteins (Figure 4.22). However, the addition of zinc (at > 4 mM) caused a marked decrease in solubility of the whey proteins (Figure 5.15). This loss of protein solubility was considered to be due to specific binding of zinc to "cleft" of α-La

in WPI, which was accompanied by aggregation of this protein (Ren *et al*, 1993). In contrast, there are no known specific binding sites in α-La for iron. The binding of iron with the whey protein molecules in WPI probably resulted from weak interactions between iron and the negatively charged amino acid residues in the proteins. The aggregates formed in iron-WPI mixtures were probably small in size or the extent of aggregation was low, hence no significant precipitation was observed when iron was added to WPI.

Although the whey proteins in WPI were more sensitive to precipitation upon addition of zinc, their binding capacity was considerably lower for zinc (2 moles Zn/mole protein) than that for iron (7 moles Fe/mole protein). Also, WPI had greater number of binding sites for iron than for zinc (Table 7.1). Again, the different chemical properties of iron and zinc (e.g. ionic radius and electronegativity) are likely to affect their binding behaviour, as mentioned above for sodium caseinate.

The iron and zinc binding characteristics of the protein molecules in MPC were fairly similar. Addition of iron or zinc caused aggregation of casein micelles in the MPC, the extent of aggregation being dependent on the concentration of added iron or zinc. The binding of these cations appeared to occur preferentially to the casein fraction in MPC as compared with the whey protein fraction. Because the casein molecules in MPC are present in the form of casein micelles, the phosphoserine residues are not free but have calcium ions bound to them. It is likely that iron or zinc would displace calcium from the phosphoserine residues as the binding affinity of these cations is much higher than calcium. In addition, it is expected that these cations would be incorporated into the colloidal calcium phosphate, possibly forming iron and zinc phosphates. As with sodium caseinate, the protein molecules in MPC also had higher binding affinity for iron than zinc. The reason for this may be the same as with sodium caseinate as mentioned above.

Table 7.1. Iron and zinc binding properties of sodium caseinate, whey protein isolate (WPI) and milk protein concentrate (MPC).

Milk Protein Products	Cations Added					
	Fe			Zn		
	Amount of iron bound to soluble protein	n	Log K_{app}	Amount of bound zinc to soluble protein	n	Log K_{app}
Sodium caseinate	~9 moles/mole protein (~23 mg/g protein)	14	5.3	5 moles/mole protein (14 mg/g protein)	6	4.84
WPI	7 moles/mole protein (19 mg/g protein)	8	3.6	2 moles/mole protein (7 mg/g protein)	3	3.29
MPC	45 mg/g protein			25 mg /g protein		

Changes in pH generally affect the complex formation between metal ions and proteins as hydrogen ions compete with the metal ion for binding to protein. In the case of sodium caseinate, the decrease in the amount of iron or zinc bound to caseinate with decrease in pH was due to a change in the ionisation state of the phosphoserine groups and below pH 5.0, aggregation of casein molecules limits their ability to bind these cations. The binding of iron appears to be somewhat less sensitive to pH changes than the binding of zinc. This may be due to the presence of co-ordination link between iron and casein molecules, as well as ionic links, but this co-ordination link is probably not present between zinc and casein molecules.

A decrease in pH from 7.0 to 2.5 caused a decrease in the binding ability of WPI for both iron and zinc, although the solubility of the whey proteins was not affected by the decrease in pH.

The effects of pH on the interactions of iron and zinc in MPC are more complicated in that the CCP begins to dissolve with a decrease in pH, causing major structural changes in the casein micelles. Consequently, the binding of iron and zinc to MPC decreases

with a decrease in pH. As in the case of sodium caseinate, caseins begin to aggregate and precipitate out of solution as their isoelectric point is approached.

There were no significant differences in the effects of ionic strength on the ability of caseins, either in their soluble (i.e. in sodium caseinate) or micellar form (i.e. in MPC) to bind iron and zinc. The affinity of caseins for iron and zinc is much higher than their affinity for sodium ions hence binding competition may not occur even when the ionic strength of the medium is increased.

In conclusion, caseins, in general, are able to bind more cations than the whey proteins, due mainly to the presence of phosphoserine residues in casein molecules. Among the three milk protein products, MPC was able to bind the most cations compared to sodium caseinate and WPI. The ability of MPC to bind more cations than sodium caseinate is probably due to the different structure of the casein molecules in these milk protein products. Caseins in sodium caseinate are present in monomers and small complexes whereas in MPC, the casein molecules consist of casein micelles bound together by colloidal calcium phosphate. The cations in MPC can bind to both the casein micelles and also the colloidal calcium phosphate.

7.2. Recommendations for Further Work

One of the major advantages of adding cations, such as iron to milk protein products, is that the milk proteins can bind the added iron, preventing it from moving between its oxidation states, hence reducing the lipid oxidation, normally caused by addition of free iron. This means the iron–protein complexes or conjugates can potentially be used as a source of iron fortification in food products and the problem of iron-induced lipid oxidation could be minimised.

The present work clearly demonstrates the binding of iron and zinc to milk protein products and the factors that affect the binding properties. Based on these results, attempt should be made to manufacture protein–iron and protein–zinc conjugates in a

pilot plant. It is envisaged that the process will involve mixing the mineral with the milk protein in appropriate proportions (based on the results of this study). This will be followed by ultrafiltration of the mixture solution to remove the free, unbound mineral. The retentates obtained can then be evaporated and spray dried to produce conjugate powders. Alternatively, these conjugates can be incorporated into milk concentrate before drying to produce iron– or zinc–enriched milk powders. Otherwise, these conjugate powders can be dry-blended into whole milk or skim milk powder. The powdered conjugates can be sold as ingredients for incorporation into other food products, such infant formulae, cheese, yoghurt, ice cream and confectionary products. The possible outline for the production of these conjugate is shown in Figure 7.1.

However, before a commercial production of these mixtures is done, some recommendations for further work are suggested:

- Because the addition of iron or zinc causes precipitation of milk proteins, especially the caseins, it is recommended to add these cations at a concentration below the critical concentrations to obtain "soluble" mineral–protein mixtures.

- It is recommended that the pH of solution be carefully monitored, as the binding of iron and zinc to proteins is markedly influenced by pH changes. At low pH values, the protein solubility is low, but the solubility of iron and zinc salts is high. The reverse is true at high pH values. Therefore, a careful pH manipulation is required to obtain optimum binding.

- The lipid oxidation test in the present study was carried out using a model system of linoleic acid emulsion. Because of the more complex structure of fat in food products, further investigation of lipid oxidation in a model food system containing milk fat and/or vegetable fats should be undertaken. Ultimately, studies will be needed to investigate the effects on lipid oxidation of adding iron–milk protein conjugate as compared with iron salts in a real food product.

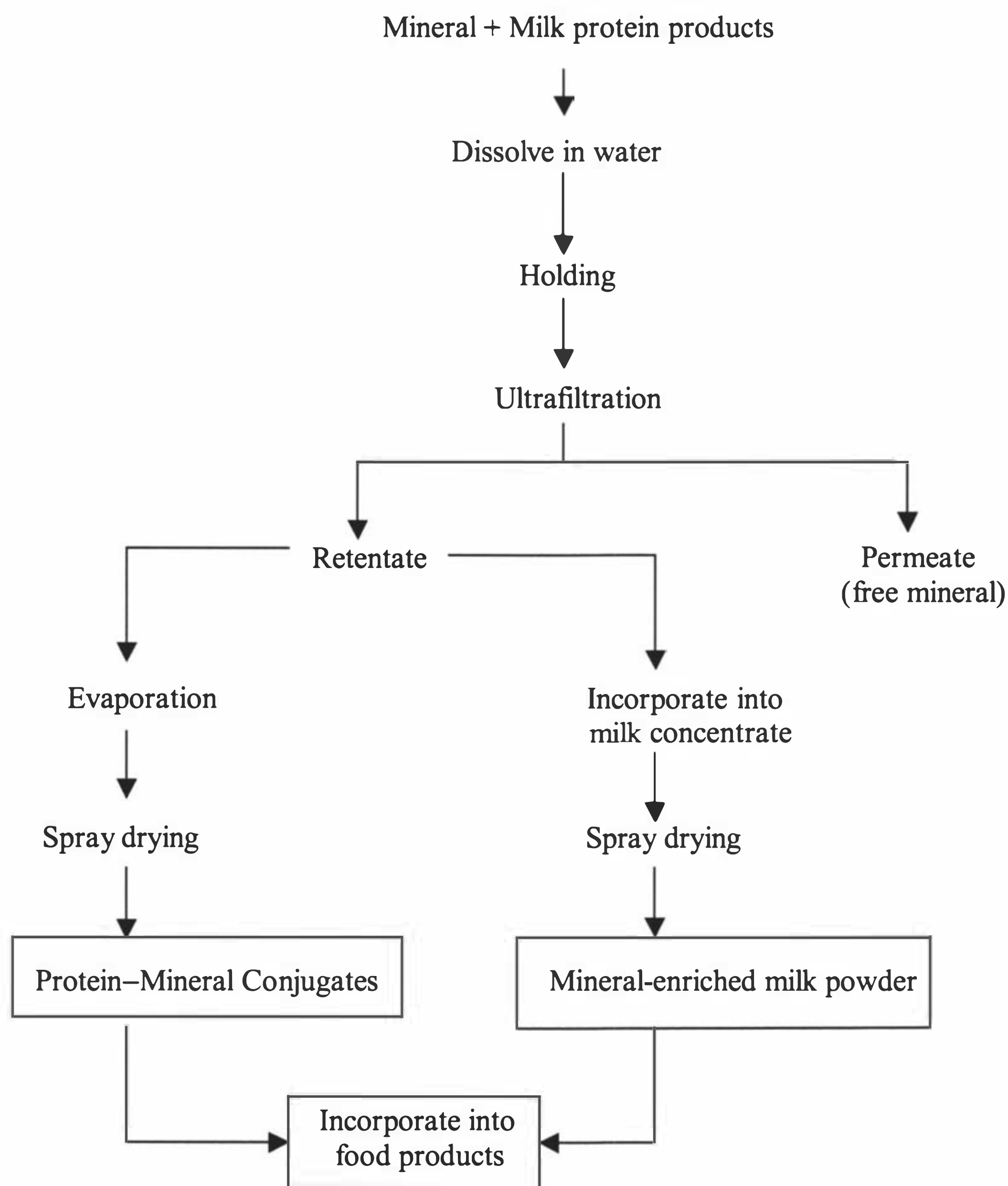


Figure 7.1. Possible outline for production and utilisation of mineral–protein conjugates.

- It is recommended to explore the sensory attributes and organoleptic properties of selected food products containing iron– or zinc–protein conjugates. This is important because addition of the cations to milk proteins may alter their functional properties.

- It is recommended that the bioavailability of the iron– or zinc–protein conjugates be determined and compared with other sources of iron (mainly low molecular weight compounds).

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