IRON BINDING PROPERTIES OF WHEY PROTEIN, CASEIN, SOYA PROTEIN AND EGG ALBUMEN

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ABSTRACT

Iron binding properties of whey protein, casein, soya protein and egg albumen were investigated in aqueous dispersions using centrifugation and ultrafiltration techniques. Protein-iron mixtures were centrifuged at 10,800 *g* for 20 min and iron that co-sedimented with protein was considered to be bound to the insoluble protein fraction. The supernatants were ultrafiltered to obtain iron bound to the soluble protein fraction.

Both the soluble and insoluble fractions of each protein were shown to bind substantial quantities of iron from ferrous sulphate. The amount of iron bound/g to the insoluble fraction of the protein was highest for casein (87 mg) followed by albumen (80 mg), soya protein (66 mg) and whey protein (63 mg). A similar trend was observed for the soluble fraction; casein bound 74 mg iron/g protein followed by albumen (68 mg), soya protein (54 mg) and whey protein (12 mg). This binding was markedly influenced by pH of the protein-iron mixtures in the range 2 - 7.

The binding data was analyzed using the Scatchard equation to obtain binding constants (k) and the number of binding sites (n). The n values obtained were ~ 2 (whey protein), 13 (casein), 200 (soya protein) and 42 (albumen). The values obtained for the binding constants were ~ 11 (whey protein), 5 (casein), 3 (soya protein) and 1 (albumen). Thus soys protein had the highest number of binding sites and whey protein had the greatest affinity for iron.

Solubility of each protein was dependent on pH and it generally decreased with increase in iron concentration.

The effects of chelating agents (citric acid and ascorbic acid) on the iron binding properties of the four proteins were also examined. Addition of citric

acid and ascorbic acid increased the solubilities of both protein and iron. The solubilizing effect of these two acids was dependent on the protein source, pH and acid concentration. Iron binding by both the insoluble and soluble fractions decreased in the presence of citric acid and ascorbic acid, with no significant differences between the effects of the two acids.

The effects of proteins and protein digestion products on *in vitro* iron availability were studied. Ferrous iron complexes with protein were prepared and subjected to simulated gastrointestinal digestion followed by measurement of soluble iron. The *in vitro* availability of iron was in the order of 26% (soya protein), 16% (casein), 14% (albumen) and 10% (whey protein). When citric acid and ascorbic acid were added prior to enzymatic digestion the availability of iron increased to 63% (soya protein), 36% (albumen), 31% (casein) and 22% (whey protein).

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CHAPTER 1

INTRODUCTION

In an effort to decrease the high incidence of iron deficiency anaemia, the food industry has been fortifying foods with iron. The continued presence of anaemia indicates that iron-fortified foods are not reaching those groups at risk in sufficient quantities to improve iron status, or that iron sources used are not as available as assumed. Technical problems are also inherent in iron fortification due to many reactions which are catalyzed by iron or in which iron is a reactant.

The solubility and chemical reactivity of an iron salt determines the kind and extent of reaction when added to a food system. Nonheme iron when added to a food system tends to form complexes with other food components. The reactivity and the solubility of the complex formed as a result of the interaction between food component and iron determines whether the food component has a inhibiting or enhancing effect on iron availability. The release of iron from these complexes depends on various factors. These factors include binding affinity of the food components for iron, digestibility of the food components, presence of reductants and accepting ligands and pH. Food components such as ascorbate, citrate, and simple sugars has been shown to contribute substantially to the release of the iron from food (Miller and Schricker, 1982).

For several decades, nutritionists have suggested that dietary protein sources influence iron bioavailability. As proteins are known to interact with iron in ways which increase or reduce availability of iron, the nature of protein-iron complex formation in foods is one of the factors which needs consideration when choosing foods to fortify. A general observation has been that meats enhance non-heme iron uptake, whereas plant, milk and egg protein depress absorption of iron from meal (Layrisse *et al.*, 1969; Cook and Monsen, 1976). Rate of protein digestion and the ability of these digestion products to chelate

iron have significant impact on iron availability. Several amino acids have been found to enhance iron uptake from iron salts when given concomitantly. The chelating ability of amino acids has been implicated in this enhancing effect. Hence it can be assumed that protein bound iron may be more biologically available than free iron salts due to the presence of absorption enhancing amino acids. Contradictory findings about the effects of proteins on iron absorption and lack of knowledge of the exact mechanism of iron absorption indicate that further research needs to be done in this area.

This study investigates the iron-binding properties of four proteins; whey protein isolate (WPI) casein, soy protein isolate (SPI) and egg albumen to bind iron from ferrous sulphate and the effects of iron concentration, pH and chelating agents on this binding. The *in vitro* availability of protein bound iron was also examined.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Chemistry of proteins

Proteins are made up of some twenty different amino acids. A variety of covalent bonds and other forces are responsible for the exact arrangement of these amino acids in proteins (Klotz, 1970). Covalent bonds are responsible for the skeleton or backbone of protein structure. The secondary and tertiary structures are stabilised by intramolecular disulphide bonds, ionic bonds, hydrogen bonds and hydrophobic interactions (Haschemeyer and Haschemeyer, 1973; Cheftel *et al.*, 1985). Ionic or salt bonds result from the interaction between acidic and basic polar side chains of lysine, arginine, histidine, aspartate and glutamate residues (Jones, 1964). Hydrogen bonds may occur among various potentially eligible groups such as, hydroxyl, amino, carbonyl and amide groups. Hydrogen bonds in protein structure determine the exact configuration of proteins (Pimentel and McClellan, 1960). The energy of these polar bonds (hydrogen bonds) is considerably less than that of ionic bonds. When the non polar surfaces of protein molecules are in contact with water, the hydrophobic effect tends to compress the protein molecules leading to the formation of aggregates. This phenomenon is called hydrophobic interaction (or bonding). The hydrophobic interactions are collective over the entire region of the protein, hence result in a strong intermolecular interaction (Timansheff, 1964). The structural forces in the proteins are not only responsible for imparting a particular configuration to proteins but they also determine the type of interactions between protein and other molecules. These intramolecular bonds can be disrupted by heat or chemical agents, leading to unfolding and, hence, denaturation of protein molecule. The three-dimensional structure of proteins is designated conformation. The polypeptide chain assumes the conformation most thermodynamically stable for the environment in which it finds itself, and in many cases, that conformation is partially fixed by the formation of disulphide bridges (Walstra, 1983).

2.1.1. Whey proteins

The term whey proteins refers to milk proteins remaining in the serum (or whey) after precipitation of the caseins from milk at pH 4.6 at 20°C. The major protein constituents of whey include β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins and proteose peptones (Table 1). There are also several minor whey proteins including lactoferrin, glycoprotein and blood transferrin.

Most of the whey proteins are compact globular proteins, ranging in molecular weight from 14,200 to 66,000 (Table 1). These proteins fold intramolecularly, burying their thiol groups (-SH) and disulphide bonds (S-S) as well as most of their hydrophobic residues, so that extensive self-association or interaction with other proteins does not occur.

Table 2.1: Concentration and properties of proteins in whey

Proteins	Concentration (g/litre)	Proportion of total protein (%)	Isoelectric point (pH)	Molecular weight (daltons)
β-lactoglobulin	3.0	50	5.3-5.5	18.3×10^3
α-lactalbumin	0.7	12	4.2-4.5	14×10^3
Immunoglobulins	0.6	10	5.5-8.3	15×10^3-1×10^6
Serum albumin	0.3	5	5.1	66×10^3
Proteose-peptones	1.4	23	--	4.1-4.08×10^3
Total	6.0	100	--	--

From Walstra (1983)

β-lactoglobulin is the most abundant protein in whey and comprises 50% of total whey proteins (Evans and Gordon, 1980), with a monomeric molecular weight of 18,000. It exists as a dimer in milk. It contains two intrachain disulphide bonds (S-S) and one -SH group per molecule.

α-lactalbumin is the second most abundant protein in whey, with a molecular weight of 16,200 (Wetlaufer, 1961; Kronman *et al.*, 1964). It contains four intrachain S-S bonds but no -SH groups. It binds two atoms of calcium very tenaciously, and removal of the bound calcium renders the protein more susceptible to denaturation by heat (Walstra, 1983).

2.1.1.1. Whey protein isolates (WPI) and Whey protein concentrates (WPC): WPI and WPC are commercial products containing high concentrations of whey proteins, usually in a substantially undenatured state (Evans and Gordon, 1980). Most of the WPC is manufactured by industrial scale ultrafiltration and diafiltration (UF/DF) technology, a substantial amount is also manufactured by ion exchange adsorption (IEA) technology. Whey is subjected to pretreatments that utilize low temperatures and low pHs, along with calcium solubilization or chelation to prevent formation of insoluble and colloidal calcium phosphate complexes during protein fractionation or concentration. The pretreated whey is further processed by UF/DF or by IEA and spray dried as WPC's. WPI is manufactured by an ion exchange fractionation of whey. Cellulosic and silica based ion exchangers have been used on small and large scale production of WPI (Palmer, 1982). Protein isolation from whey using the cellulosic ion exchanger takes place at pH 8.0 for maximum protein adsorption, and maximum adsorption using cation-exchanger CM-C takes place under acidic conditions around pH 3.0. Elution of the adsorbed protein takes place by bringing the pH to 5.0 and increasing the ionic strength with salt. The eluate, which comprises a dilute solution of almost pure protein, is concentrated and dried by freeze or spray drying to yield a protein isolate containing over 90% protein.

WPI's and WPC's are highly soluble over a wide range of pH. They are able to form gels on heating at about 5% concentration and above, they exhibit good fat and water binding properties, they are good aerating agents and are excellent nutritional materials (Evans and Gordon, 1980).

Table 2.2: Composition of whey protein isolate

Components	%
Protein	90.4
Moisture	6.2
Ash	0.5
Fat	0.9

From Evans and Gordon (1980)

2.1.2. Caseins

The caseins are defined as those phosphoproteins which precipitate from raw milk upon acidification to pH 4.6 at 20°C. The caseins consist of four distinct proteins : α_{s1}-, α_{s2}-, β- and κ-caseins in approximate proportions of 38%, 10%, 36% and 13%. All four proteins have distinct amphiphatic character with separate hydrophobic and hydrophilic domains; this character is enhanced in α_{s1}-, α_{s2}- and β- caseins by phosphorylation of seryl and threonyl residues. In addition, high content and uniform distribution of proline residues along the polypeptide chain result in low levels of secondary and tertiary structures. At temperatures below 40°C, α_{s2}- casein is most sensitive to calcium, and is precipitated by calcium concentrations above 2 mM while α_{s1}- precipitates in the range of 3 - 8 mM, β- casein precipitates in the range of 8 - 15 mM calcium and is quite soluble below 5°C. The caseins interact among themselves and with salt ions to form micelles (Lindquist, 1963) as well as with whey proteins to form complexes (McMeckin and Groves, 1964).

Table 2.3: Composition and properties of caseins

Proteins	Conc (g/l)	% of total casein	Isoelectric pH	M.W (daltons)
α_{s1}-	10	39	4.9	23,614
α_{s2}-	2.6	12	5.3	25,230
β-	9.3	36	5.2	23,983
λ-	3.3	13	5.5	19,023

After Swaiswood (1989).

2.1.2.1. Caseinates: Commercial caseinate is manufactured from skim milk by precipitating the casein and solubilizing the acid casein in sodium hydroxide. Acid casein curd is minced to disintegrate the curd and is mixed with water at 40°C, passed through a colloid mill into which sodium hydroxide is pumped, the slurry emerging from the mill has a smooth consistency with pH of 6.6 - 6.8. The slurry is efficiently mixed, transferred to a vat where the mixture is vigorously agitated and heated. In the process, the temperature of the slurry is raised from 45°C to 95°C. The solution is pumped from the vats to the spray drier. During caseinate production, care is taken to minimize the time for which casein solution is held at high temperatures, since brown colouration may occur, and the time for which the casein solution is exposed to high pH during dissolving as it may lead to development of off-flavours. Spray drying procedures are adjusted to obtain a product with 5% or less moisture content. Dry sodium caseinate contains about 90 to 94% protein, 3 to 5% moisture, 6 to 7% ash. Casein is widely used in food products as a protein supplement in dietetic and bakery products, and imitation milk.

Table 2.4: Composition of sodium caseinate

Components	Percent
Protein	90-94%
Moisture	3-5%
Ash	6-7%
Fat	0.7-1%

From Fox and Mulvihill (1989)

2.1.3. Soy proteins

Soy proteins are storage proteins extracted from soybeans. Soy bean proteins consist of mainly four fractions, designated as 2S, 7S, 11S and 15S, based on their sedimentation coefficient (Wright, 1988). They have a relatively high solubility in water or dilute salt solutions at pH values above or below the isoelectric point.

2.1.3.1. Isolated soy protein: Isolated soy protein is defined as the major proteinaceous fraction of soybeans prepared from high quality, cleaned, dehulled soybeans by removing the non-protein components. The starting material for isolated soy protein production is defatted soy flakes or flour with high protein dispersibility. The extraction process involves wetting the soy flakes with a proper amount of water at a controlled temperature and mixing the necessary amounts of high quality food grade chemicals for a defined length of time. The pH is closely controlled throughout this step in the process, as it is critical for the overall yield. After the protein is solubilized, it is separated from the insoluble polysaccharides and crude fibre by centrifugation. The protein extract contains the soluble carbohydrates and the major protein fractions. The pH of the extract is adjusted to approximately 4.5, resulting in the precipitation of the major protein fractions. The precipitated protein is referred to as soy protein curd, which

is spray dried producing an isoelectric type soy protein isolate. Sulphur containing amino acids, methionine and cysteine are the limiting amino acids in soy protein isolate. Soy protein isolate contains nutritionally significant minerals, such as calcium, iron, copper, phosphorus, zinc and sodium (Torun, 1979).

The major applications of soy protein products are in processed meat and fish products, bakery products, dairy-type products, infant formulas, protein supplements, hospital feeding and meat analog products (Waggle, 1978). According to Seal (1980), isolated soy protein is one of the most suitable sources of protein for slimming food products because of its sensory qualities, favourable amino acid composition, high protein content with low non-protein calories. It has a good dispersibility, suspension properties and storage stability (Waggle and Kolar, 1978).

Table 2.5: Composition of isolated soy protein

Components	%
Protein	92
Oil	0.5
Ash	4.5
Carbohydrate	0.3

From Waggel and Kolar (1979)

2.1.4. Egg albumen

Albumen or egg white consists primarily of protein with small amount of sugar and mineral on a dry basis (Froning *et al.*, 1988). According to Powrie (1977), it may be regarded as a protein system consisting of ovomucin fibres in an aqueous solution of numerous globular proteins. The major proteins present are as ovalbumin, conalbumin, ovomucoid, lysozyme and globulins

(Fronning, 1988); Ovomucin, ficin, ovoinhibitor, ovoglycoprotein and avidin are other proteins which are present in lesser amounts.

Table 2.6: Proteins of egg albumen

Protein	% of albumen	Isoelectric pH	M.W (daltons)
Ovalbumin	58	4.5	45,000
Conalbumin	12	6.1	76,000
Ovomucoid	11	4.1	28,000
Ovomucin	3.5	4.5-5.0	$5.5\text{-}8.3 \times 10^6$
Lysozyme	3.4	10.7	14,300
Globulins	4.0	5.5	$3.0\text{-}4.5 \times 10^4$
Avidin	0.05	10	68,300

From Powrie (1977)

Ovalbumin is the principal protein in egg albumen, making up 54% of the total protein present and having a molecular weight of 45,000 (Fronning, 1988). It is classed as phosphoglycoprotein since carbohydrate and phosphate moieties are attached to the polypeptide (Powrie, 1977). A molecule of ovalbumin contains four -SH groups with three weakly reactive SH groups in native state and four reactive groups after denaturation (Fernandez-Deiz, 1964). According to Powrie (1977), there are two disulphide bonds per molecule of protein.

Conalbumin is also referred to as ovotransferrin. This glycoprotein has been shown to bind iron and is an anti-bacterial agent in the egg (Fronning, 1988). It has a molecular weight of 76,000 and contains no phosphorus and -SH groups (Clark, 1963). Di- and trivalent metallic ions are bound firmly by conalbumin. Two atoms of Fe (III), Al (III), Cu (II) and Zn (II) per molecule

of protein form stable complexes above pH 6.

Ovomucoid is a heat-resistant glycoprotein containing 20-25% carbohydrate, exhibiting anti-trypsin activity and having a molecular weight of 28,000 and isoelectric point between 3.9 and 4.3 (Powrie, 1977). Ovomucin is a glycoprotein which provides gel structure to thick albumen (Froning, 1988).

Lysozyme is a bacteriolytic agent with a molecular weight of 14,300 (Canfield, 1963) and an isoelectric point of 11.7 (Froning *et al.*, 1988). It contains 129 amino acid residues and four disulphide bonds (Canfield and Liu, 1965). It consists of 2 or 3 three components which can be separated by cation exchange chromatography (Powrie, 1977).

Avidin is a basic glycoprotein with four subunits with a combined molecular weight of 70,000 consisting of four identical polypeptide chains, each containing 128 amino acid residues (Green, 1964). It binds biotin; about three moles of biotin are bound to one mole of avidin (Powrie, 1977).

Globulins have three fractions G_1, G_2 and G_3. The molecular weight of this fraction of albumen is 35,000 and has an isoelectric point of 5.5. Flavoprotein binds riboflavin (Rhodes, 1958) and the fraction which does not contain riboflavin is termed apoprotein (Powrie, 1977). It has molecular weight of 36,000 and an isoelectric point between 3.9 and 4.1

2.1.4.1. Separation of egg white protein: Egg white and egg yolk are separated mechanically by breaking the shell and collecting albumen and yolk in different containers. Large scale production plants have egg breaking machines which makes it possile to produce whole eggs, egg yolk and egg white simultaneously. The liquid egg white thus obtained is dried, in drying of egg white, moisture is removed from liquid until only a solid portion, with a small quantity of moisture remains.

Spray drying is the most important method of producing dried egg products. In spray-drying, the liquid is finely atomized into a stream of hot air. Because of the enoromous surface created by atomization, evaporation of water is very rapid. The air used for drying is filtered by suitable means to remove undesirable dust. It is heated to a temperature of between 121°C and 232°C and moved to the spray drying chamber by a fan. The liquid is moved to the drying chamber by a suitable pump and is atomized into the hot air stream by one of several types of atomizing devices. The powder formed separates from the drying air in the drying chamber and also in a separating device which is a part of, or separate from, the spray-drying chamber. The air is then removed from the system by an exhaust fan. The dried product is removed from the dryer, cooled, and sifted before packaging.

Pan drying, freeze drying and belt drying are other methods of drying liquid egg products. Flake-type egg white products are made by means of pan-drying. In freeze-drying, water is removed from the product while it is in the frozen state. This is accomplished by freezing the product and then subjecting it to a very high vaccum. The cost of freez drying is higher than other methods of drying, hence, its commercial applications are rather limited. In belt drying, the liquid is spread as a thin film on a continious aluminium belt moving through a hot air stream.

2.2. Binding of proteins to metal ions

Proteins have the ability to bind several molecules of metal ions. 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 sites to understand the biochemical systems (Freifelder, 1982). 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 protein 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 observed in ion and proton binding to proteins.

2.2.1. Determining binding sites and binding constants

If a protein, **P**, binds ligand, **A**, at **n** sites (sites on **P** which may be chemically equivalent or different), then the equilibria obtained may be represented by the following generalized equation:

$$PA_{(n-1)} + A \rightleftharpoons PA_n \qquad (1)$$

The dissociation constant K_d for this reaction can be defined as

$$K_d = \frac{[P]\,[A]}{[PA]} \qquad (2)$$

Equation (2) indicates that the fraction of ligand bound is affected by the value of [P] and [A]. The number of moles of ligand bound to one mole of macromolecules; $\bar{v}$ is defined as follows:

$$\bar{v} = \frac{[A]_{bound}}{[P]_{total}} = \frac{[PA]}{[P] + [PA]} \qquad (3)$$

To evaluate $\bar{v}$, a particular concentration [A] of ligand is added to a known concentration [P] of the macromolecule and then either the concentration of bound ligand [PA] or of unbound ligand [A] is measured. Combining equations (2) and (3) yields

$$\bar{v} = \frac{[A]}{k_d + [A]} \qquad (4)$$

which is known as the *Langmuir isotherm*. In order to plot a straight line graph, the equation can be written in the following two forms:

$$\frac{1}{\bar{v}} = \frac{k_d}{[A]} + 1 \qquad (5)$$

$$\frac{\bar{v}}{[A]} = \frac{1}{K_d} - \frac{\bar{v}}{K_d} \qquad (6)$$

If all binding sites are identical and independent, the relevant equations similar to equation (5) and (6) are the following:

$$\frac{1}{\bar{v}} = \frac{1}{n} + \frac{K_d}{n[A]} \qquad (7)$$

$$\frac{\bar{v}}{[A]} = \frac{n}{k_d} - \frac{\bar{v}}{K_d} \qquad (8)$$

in which K_d is an average dissociation constant and n is the number of binding sites. When equation (7) is used, a plot of $1/\bar{v}$ versus 1/[A] gives a straight line, having a slope of k_d/n and a y-intercept of 1/n. Equation (8) is called the scatchard equation, a plot of $\bar{v}/[A]$ versus $\bar{v}$ gives a straight line, having a slope of $-1/K_d$ and an x-intercept of n/K_d. An advantage of using this method of data analysis is that if the binding sites are not both identical and independent, neither of the plots will give straight lines. Another special value of scatchard equation is that, the value of n can be read directly from the graph.

2.3. Interactions of iron with food proteins and their effect on absorption

2.3.1. Milk proteins

Consideration of addition of iron to milk as a way of combating iron deficiency anaemia led to the investigations of binding of added iron to various fractions of milk (Demott and Dincer, 1976). Allan (1950) and King *et al.* (1959) thought that both natural and added iron was anionic and was bound to fat

globule membrane. Basch *et al.* (1974) and Demott and Park (1974) found that added iron was associated largely with protein, primarily with casein which agrees with the findings of Demott and Dincer (1976) who showed that about 85% of added iron was bound to casein fraction and 9% to the whey protein fraction. This led to the investigation of the casein fractions to which the added iron is bound. It was found that, of the 85% of added iron bound by casein fraction, 72%, 21%, and 4% was associated with α_s-, β-, and κ-caseins, respectively. α_s-Casein has been reported to be the principal iron binding protein in milk (Hegenauer *et al.*, 1979).

Nelson and Potter (1979a) investigated the binding of iron to food proteins and reported that casein bound considerable amount of iron; 8.7 mg iron/g protein was found to be bound to the insoluble fraction. Results of a study on the binding of supplemental iron to casein micelles by Hegenauer *et al.* (1979) showed that the casein micelle was the principal sequestrant of iron added to milk and at low iron 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. It was concluded that iron added to whole or skim milk is bound almost exclusively by the phosphoproteins of the intact casein micelle. Carmichael *et al.* (1975) attributed the strong affinity of caseins for iron to the presence of clustered phosphoserine residues in these phosphoproteins.

Although iron content of milk is low, there have been many suggestions for the use of milk as an iron fortification vehicle (Carmichael *et al*, 1975; Ranhotra *et al.*, 1981). Klavins *et al.* (1961) found increased iron absorption in the presence of casein, but when the meal constituted of casein and 60% fat they were unable to conclude which factor led to increased iron absorption. Carmichael *et al.* (1975) found that iron from ferric-casein was as available as from low molecular weight, soluble ferric nitrilotriacetate (Fe-NTA) chelate. They also found that milk enhanced iron absorption from Fe-NTA. Hence these researchers concluded that milk and casein did not depress iron

absorption. However, as Fe-casein was prepared by incubating milk with Fe-NTA and revovering the casein by centrifugation; it is thus possible that significant amounts of NTA remained with the casein fraction thereby increasing iron absorption (Berner and Miller, 1985).

In an *in vitro* study, Peters *et al.* (1971) carried out simulated pepsin digestion and measured dialyzability of iron following the digestion. Milk markedly inhibited the dialysis of iron. Cook and Monsen (1976) showed that non-heme iron absorption was much greater in the presence of muscle meats than when milk or cheese replaced the meat. In two balance studies with children, milk depressed the absorption of iron from a ferric chloride solution or from a mixed diet (Sharpe *et al.*, 1950; Abernathy *et al.*, 1965). In a study of blood parameters in infants, haemoglobin values were significantly depressed when iron-poor, high-milk protein formulas were fed, as opposed to iron-poor, low-milk protein formulas (Berner and Miller, 1985). The results of these studies suggest that milk proteins depress iron absorption.

2.3.2. Soya proteins

The inhibitory effect of non purified soy protein on iron absorption has been attributed to the binding of iron by phytate associated with these proteins. Phytate forms strong complexes with protein and this complex has the ability to bind iron more tenaciously then phytate or protein alone (Clydesdale, 1982). Welch and Van Campen (1975) investigated the comparative effect of mature and immature soy seeds on binding of iron, and found that iron from immature seeds was less available, indicating that these seeds contain factors other than phytate which has the ability to bind iron. Fibre has been implicated in decreasing trace element availability in meals containing soy protein by Reinhold (1973), but the low concentration of crude fibre in soy products in general and soy protein in particular suggests that fibre is not responsible for low trace element availability from these products (O'Dell, 1979).

Schnepf and Satterlee (1983) by utilising the everted rat gut assay demonstrated that soy isolate tended to bind more iron than did the soy flours, and that soy protein has two major sites of binding for iron; one site is on the surface of the protein molecule and the other site is within the protein molecule. They found that 70% of added iron was bound which represented 19.6 mg of iron per gram of soy isolate. Nelson and Potter (1979a) reported that 17 mg of iron was bound to 1 gm of soy protein isolate, a value identical to that obtained by Schnepf and Satterlee (1983). Rizk and Clydesdale (1983) found binding of iron by soy isolate was directly influenced by pH. At pH 4, a smaller amount of iron was in soluble form (more binding) than at pH 2, suggesting that as pH increased the soluble iron was also complexed by soy protein isolate.

The results of Steinke and Hopkins (1978) using rats and Rios *et al.* (1975) on humans showed that iron from soy beans was well absorbed. Cook *et al.* (1981) performed iron absorption studies in humans fed with soy protein and their results indicated a significant inhibition on the absorption of nonheme iron. Studies by Morck *et al.* (1981) showed similar results. The results of these studies indicate that the information available on the effect of soy beans and soy protein products on iron availability is contradictory.

2.3.3. Egg proteins

Nelson and Potter (1979) reported that the soluble and insoluble fractions of egg albumen had the ability to bind ferric and ferrous. Maximum amount of iron bound by the insoluble fraction was 6.3 mg and 14.4 mg for 1 g protein at 25°C and 60°C respectively. They reported that albumen, incubated with ferrous iron, decreased in solubility over the incubation at both the temperatures. They attributed this to the denaturation of proteins by iron salts and coagulation at 61°C (Nelson and Potter, 1979a). Conalbumin, a protein in egg albumen, has the ability to combine with metal ions (Alderton *et al.*, 1946) and this binding may be responsible for decreased iron availability in the presence of egg albumen. Warner and Weber (1953)

suggested that conalbumin contains a hydroxylamino group which binds iron. But experiments by Fraenkel-Conrat (1950) on hydroxylamino derivatives of proteins indicate that conalbumin does not contain a stable group of this kind. Since no unusual groups with metal binding properties have been found in conalbumin, it is probable that the specific properties of the binding sites are a result of unique steric arrangement of the several groups comprising the site (Warner and Weber, 1953).

Eggs, although high in iron content, have long been considered poor sources of iron (Chodos *et al.*, 1957; Callender *et al.*, 1970). The human study data of Rossander *et al.* (1979) indicated that total absorbed iron was not significantly increased by the addition of egg to a breakfast meal even though the egg supplied 1.3 mg iron of a total of 4.1 mg iron in the meal. The non-availability of iron has been attributed to binding by phosvitin (Hegenauer *et al.*, 1979). Egg albumen was shown to inhibit non-heme iron absorption in studies by Monsen and Cook (1979); when subjects were fed meals composed of semisynthetic components, egg albumen strongly inhibited absorption of nonheme iron (Fig, 2.1).

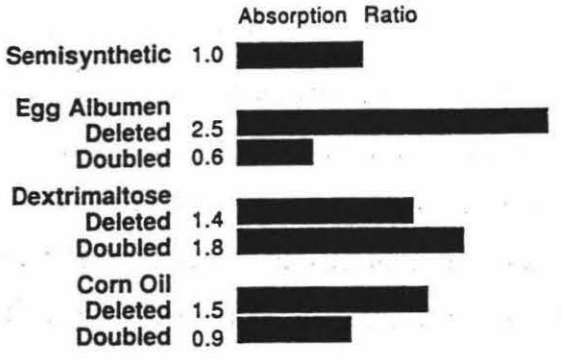


Fig 2.1 Absorption of nonheme iron from semisynthetic meals. Absorption ratios compare % absorption of nonheme iron from test meal to % absorption of nonheme iron from semisynthetic meals. *(From Monsen and Cook, 1979).*

2.4. Effect of chelating agents on the binding of iron to food proteins

Chelating agents can disrupt the bond between iron and iron binding substances, as they have a greater affinity for iron (Clydesdale, 1988). If the chelating agent forms a soluble complex with iron and if its stability constant is greater than that of another iron binding component, it has an enhancing effect on iron absorption. The ability of the chelating agent to disrupt the bond between iron and protein depends on the strength and the binding sites involved.

2.4.1. Ethylenediaminetetraacetate (EDTA)

Research shows that EDTA mobilizes zinc, manganese, and iron from other constituents in the diet, such as dietary fibre and some proteins (Layrisse *et al.*, 1976; Viteri *et al.*, 1978; Martinez-Torres *et al.*, 1979). These chelated elements were in soluble form hence more available (Clydesdale, 1988). Studies by Satterlee and Schnepf (1983) on iron binding showed that EDTA was able to successfully chelate the iron and prevent it from binding to the soy protein isolate. EDTA at a concentration of 0.01 M formed a soluble iron chelate which was able to solubilise iron in the presence of soy protein isolate.

2.4.2. Ascorbic acid

The enhancing effect of ascorbic acid on the absorption of iron from meals has been demonstrated by El Hawry *et al.* (1974), Monsen and Cook (1979) and Gilloly *et al.* (1984). The enhancing effect may be related to both its reducing effect, preventing the formation of insoluble iron hydroxide, and to the formation of soluble complexes with ferric ions, which preserve iron solubility in the duodenum (Conrad and Schade, 1968; Conrad, 1970). In the presence of other dietary components which bind iron, the absorption enhancing effect of ascorbic acid depends on its ability to form a more stable bond with iron.

Satterlee and Schnepf (1983) investigated the effect of chelating agents on soy protein-iron binding and found that ascorbic acid (0.01 M) was able to reverse some of the binding of iron by soy protein isolate. Morck *et al.* (1982) found

similar effect *in vivo*, and suggested that it may be due to the ability of ascorbic acid to form low molecular weight chelates. Many investigators have demonstrated the importance of considering the ascorbic acid to iron ratio in both *in vivo* and *in vitro* studies (Lynch and Cook 1980; Kojima *et al.*, 1980; Bothwell *et al.*, 1982). Rizk and Clydesdale (1983) found that there was a significant increase in percent soluble iron when the ascorbic acid to iron ratio was 50:1, when compared with the ratio 25:1. They observed that addition of ascorbic acid doubled the amount of iron present in soluble form, but this iron (soluble) was complexed with ascorbic acid. Kojima *et al.* (1981) reported that ascorbic acid was maximally effective in solubilizing iron in a bean (pinto) suspension between pH 1.5 and 5. A similar effect was observed by Rizk and Clydesdale (1983) in their study on binding of iron to soy isolate, they noted drastic decreases in the enhancing factors (low molecular weight chelates) at pH 6.

2.4.3. Citric acid

Citric acid has been identified as the low molecular weight Zn-binding ligand in human milk by Hurley and Lonnerdal (1982). Citric acid has been found to both inhibit and enhance iron absorption, depending on the meal (Hallberg and Rossander, 1984). Lyon (1984) evaluated the ability of citric acid to promote mineral solubility and found that citric acid was very efficient in solubilizing iron and zinc. Several workers (Kojima *et al.*, 1981; Leigh and Miller, 1983; Rizk and Clydesdale, 1985; Nadeau and Clydesdale, 1986) have shown that citrate is effective in solubilizing iron but the effect is dependent on pH and the ratio of iron to citrate. Nadeau and Clydesdale (1986) found an increasing iron solubilizing effect in cereal samples between pH 2 and pH 6 with iron:citrate ratios between 1:0 to 1:150.

Warner and Weber (1953) examined the formation of complexes of both ferric and cupric ions with citric acid as a function of pH. In each case, they found a chelate with a metal ion:citrate ratio of 1:1. Similar ratios were found by Spiro *et al.* (1967) who also demonstrated that addition of base to a solution,

containing equimolar iron (III) and citrate at low pH, led to the formation of anionic monocitrate-iron chelate, postulated to be $FeCit^{-}$. This compound then polymerized at higher pH values. In the presence of excess citrate, polymer formation was not favoured. Hence, they concluded that excess citrate must cause the formation of low molecular weight species rather than the polymers and act as enhancer of iron absorption.

2.5. Measurement of iron bioavailability

Availability or bio-availability has been defined as the proportion of a nutrient in food which is absorbed and utilized (O'Dell, 1989), or as usefulness of nutrients to the live organism (Greger, 1989). Southgate (1989) proposed that bioavailability refers to the quantity of the nutrient present in the diet either in the form that can be transported across the intestinal mucosa, or to the ingested form capable of being transformed into transportable forms that are absorbed and utilized in normal metabolism.

Both *in vitro* and *in vivo* studies can be used to determine bioavailability. *In vivo* techniques include:

1) extrinsic tagging, wherein a test diet is spiked with an exogenous ^{59}Fe label and the percent absorption of the label is used as an indicator of iron absorption from the meal (Consaul and Lee, 1983)
(2) intrinsic tagging, wherein a food is grown in radiolabelled solution, or an animal is injected with radioactive iron, and the percent absorption of label is the measure of iron absorption from the food (Consaul and Lee, 1983)
(3) balance studies
(4) haemoglobin repletion studies
(5) changes in blood parameters, such as serum ferritin or haemoglobin.

In vitro techniques involve simulation of gastric or gastrointestinal digestion using purified peptic and/or pancreatic enzymes. There are two approaches for *in vitro* estimation of bioavailable iron. One approach is to measure

"ionizable" or "ionic iron" in foods. This is done by determining the fraction of the total iron in a food that reacts with complexing agent such as bathophenanthroline to form a chromogen, which can be quantitated spectrophotometrically (Narasinga Rao and Prabhavathi, 1976). A second approach is to measure the soluble iron released by digestion. Two methods of separating soluble iron from insoluble iron include dialysis and centrifugation (Manis and Schachter, 1962). Ultrafiltration is a third means of separating complexed iron from free iron (Satterlee and Schnepf, 1983).

The advantages of *in vitro* methods are low cost and speed, reduced variability compared to *in vivo* methods (variability caused by differences in iron status of animals and humans is avoided) and ability to precisely control conditions during the determinations. Disadvantages of *in-vitro* method include uncertainties over the use of an artificial system, less than exact duplication of *in vivo* conditions and inability to account for effects of active transport and brush border binding proteins. *In vitro* techniques can be used to rank foods into broad categories of iron availability, to study iron chemistry during the digestive process, or to study the impact of addition or deletion of certain foods. Animal and human studies can be reserved for those factors which have considerable impact on iron availability. *In vitro* studies can be used as a tool to screen out factors that are unlikely to have major impact on iron availability (Van Campen, 1983).

2.5.1. Variables to be controlled in in-vitro studies

The primary determinants of food iron availability are the extent of iron release from food and solubility. The molecular weight and stability of the complexes formed from the released iron, pH, enzyme concentration and digestion times (Miller and Schricker, 1982), are the factors which influence these determinants, hence these variables need to be controlled in *in vitro* studies.

2.5.1.1. pH: Gastrointestinal pH has a significant effect on the bioavailability of iron. Accroding to Miller and Schicker (1982) ingestion of a meal by human subjects increased gastric pH from about 2 to 5, the peak pH of 5 was reached rapidly. It then gradually lowers with time and is stabilized at 1.5 to 2 by the end of second hour. This suggests that stomach acid titrates its contents to an acid pH and that stomach pH's following different meals should be similar. It, therefore, can be assumed that adjustment of pH to 2 prior to *in vitro* pepsin digestion would approximate the *in vivo* situation (Miller and Schricker, 1982). As the food products reach the duodenum bicarbonate secreted by the pancreas neutralizes the stomach acid from pH ~2 to pH 7.2 (Narsinga Rao and Prabhavathi, 1979). Miller and Schricker (1982) suggested that pH of the pepsin digest should be adjusted to pH 7 prior to addition of pancreatin, in order to generate *in vivo* conditions. As the iron is released from food in the acidic environment of the stomach, ligands released in the digestion process combine with the iron to form chelates. These chelates inhibit polymerisation and precipitation of iron as the stomach contents are neutralized in the duodenum. This suggests that pH adjustment step in an *in vitro* simulation is a critical step (Miller and Schricker, 1982).

2.5.1.2. Concentration of digestive enzymes: A large range of concentrations of digestive enzymes used in *in vitro* studies have been reported. Akeson and Stahmann (1964) used 15mg pepsin and 40mg of pancreatin per gram of protein. Narasinga Rao and Prabhavathi (1978) used pepsin concentrations ranging from 12 to 60mg per gram of food. They reported no difference in iron release when this range of pepsin concentration was used. Lease (1976) used 20mg of pepsin per gram of food. Hazell *et al.*(1978) used 10mg of pepsin and 10mg of pancreatin per gram of protein. While different concentrations of enzymes produce different rates of digestion, the actual enzyme concentrations are not critical provided they are precisely duplicated when comparisons between foods or meals are being made (Miller and Schricker, 1982).

2.5.1.3. Digestion time : Rates of passage of digesta are determined by several interacting factors including the osmolarity of the meal, the relative amounts of liquid and solid in the meal, the size of the meal and the carbohydrate, protein, and the fat content of the meal (Fein, 1980). Narasinga Rao and Prabhavathi (1978) showed in an *in-vitro* system that iron released from food was the same when pepsin incubation times were varied from 50 to 180 minutes.

According to Miller and Schricker (1982), it is best to select the digestion time that produces significant digestion, but care must be taken to use identical digestion times when comparisons are being made between foods or meals.

2.5.2. Procedures for in-vitro technique

In vitro techniques have been frequently been used to assess iron availability. Rao and Prabhavathi (1978) have used an *in-vitro* method which they found to yield estimates for iron availability similar to those obtained in human trials. In their procedure, the food or meal was first incubated with pepsin-HCl at pH 1.35. At the end of the incubation, the contents of the flask were centrifuged and the supernatant was filtered. Soluble and ionizable iron were determined in the aliquots of the filtrate at pH 1.35. In another aliquot, pH was adjusted to 7.5 with NaOH and incubated. At the end of this incubation period the contents of the flask were centrifuged, the supernatant obtained was filtered and the filtrate was used for the determination of soluble and ionizable iron. The percent ionizable iron was reported to correlate well with percent iron absorption from the same diets by adult males.

The method suggested by Miller and Schricker (1982) is similar to the method suggested by Rao and Prabhavathi (1978) except in two aspects; pH adjustment was achieved by dialysis and low molecular weight soluble iron rather than total soluble iron was used to estimate available iron.

Nelson and Potter (1979b) used a method similar to the one suggested by Rao and Prabhavathi (1978) but digestion tests were carried out in three stages; protein-iron mixture with HCl was the first stage of digestion, the second stage was incubation of protein-iron mixture with HCl-pepsin, and at the end of this incubation the samples were neutralized, pancreatin was added and incubated for 24 hr.

As the response parameter for available iron differed in different methods, there is a variability in the results, but the factors which caused significant effect on the bioavailability of iron were similar in all the studies.

2.6. Effect of solubility on iron bioavailability

Iron bioavailability is greatly influenced by solubility. The importance of solubility is indicated by the fact that in most of the *in vitro* bioavailability tests, soluble iron is measured as the available iron. Soluble salts which remain stable through the varying conditions in the gastrointestinal tract will be the most available (Clydesdale, 1983). Solubility is influenced by valency and charge density of iron, particle size, pH and complexes formed with other dietary components.

2.6.1. Valency and charge density

Iron in the ferrous state is more soluble than ferric iron, hence ferrous iron is more available (Miller and Schicker, 1982). When iron is added to food, the food environment affects the valency state. The redox potential of the system maintains or alter the valency of the added iron. When the redox potential of the system is lower than +770 then ferric iron is reduced to ferrous iron (Clydesdale, 1983).

Charged molecules do not easily cross the cellular membranes of the gastrointestinal tract. Thus, iron or iron complexes of lesser charge might be able to cross the mucosal membranes more easily (Berner and Miller, 1985).

2.6.2. Particle size

The size of the particle is inversely related to bioavailability (Shah *et al.*, 1977). The smaller the particle size, greater the solubility particularly at low pH as in the stomach prior to entering the duodenum (Coccodrilli, *et al.*, 1976).

2.6.3. pH

In a solution at low pH, iron exists as hydrates. As the pH is raised, protons are lost and hydroxides are formed in neutral and alkaline solutions. Hydrates are more soluble than hydroxides which form insoluble precipitates (Lee and Clydesdale, 1979). Hence, iron is more available when the pH of the system is acidic, availability is reduced when the pH is neutral or higher (Clydesdale, 1982). The pH also influences the degree of ionisation of iron. Ferrous iron is maximally ionised at pH 4 - 5 and ionisation of ferrous iron is greater than ferric iron, irrespective of the pH of the system (Clydesdale, 1982). Nojiem and Clydesdale (1981) showed that the ferrous state is favoured at low pH (2.7) and of the ferric state at high pH (6.2).

2.6.4. Dietary components

Iron forms chelates with other dietary components, the solubility of the chelates formed determines the availability of iron in that food environment. Iron availability from the diet is related to the concentration of soluble iron and low molecular weight complexes compared to the concentration of agents which form insoluble precipitates, macromolecules, or high-molecular-weight complexes preventing iron absorption (Smith, 1983). Ligands which form soluble complexes enhance iron absorption while formation of insoluble complexes renders iron unavailable. Ligands which reduce iron availability are called "inhibitors". A ligand which is a good enhancer of iron absorption must firstly be soluble and secondly must bind iron securely in a complex which remains stable in the gastrointestinal tract. But the bond must not be so strong that it cannot release iron to an acceptor in the intestinal mucosa. Ascorbic acid, citric acid, EDTA are enhancers of iron absorption. Some

proteins, fibre and tannins are some of the inhibitors of iron absorption.

2.7. Role of protein in the absorption of iron

2.7.1. Protein digestion

Hydrolysis of proteins in the gastrointestinal tract is accomplished by specific enzymes secreted in the gastric and pancreatic juices and by the mucosa of the small intestine.

2.7.1.1. Gastric digestion: The proteinase of the gastric juice is pepsin which hydrolyzes peptide bonds involving an aromatic amino acid (phenylalanine or tyrosine) in both proteins and polypeptides. Pepsinogen is the precursor of pepsin. Pepsin can break down proteins into amino acids, but as the food remains in the stomach for a limited time degradation of proteins results in a mixture of products which include large polypeptides and peptides. If gastric hydrochloric acid production fails to maintain gastric contents at the optimum pH (2 to 3) for peptic action, protein digestion is impaired (White *et al.*, 1959).

2.7.1.2. Proteolysis in the intestine: The acid chyme from the stomach is neutralized by bicarbonate which is a constituent of bile and pancreatic juice. The alkalinity helps in stimulating the secretion of pancreatic juice by pancreas. Pancreatic juices contain three enzymes; trypsin, chymotrypsin and carboxypeptidase. Each of these enzymes is effective in catalyzing hydrolysis of different peptide bonds in proteins, polypeptides, and smaller peptides. The successive action of the proteolytic enzymes results in the ultimate hydrolysis of dietary protein to amino acids (White *et al.*, 1959).

2.7.2. Mechanism of iron absorption

Protein may inhibit or enhance iron availability. This depends on the type of complex formed by the protein digestion products and iron. If the peptide released after digestion forms a soluble complex, and readily releases iron to the receptor then the protein has an enhancing effect. If the complex formed

is insoluble, or if a soluble complex is formed which does not release iron to the receptor then the protein has an inhibiting effect on iron absorption (Berner and Miller, 1985). This hypothesis is based on the assumption that to be available, iron must be soluble (Bothwell *et al.*, 1979; Cook *et al.*, 1982) and must be in low molecular weight form (Miller *et al.*, 1982) in the upper small intestine. There is no direct evidence for the later assumption as the receptor for the low molecular weight iron in the intestine has not been established (Berner and Miller, 1985). But iron absorption studies show that iron from low molecular weight complexes such as Fe-fructose (Bates *et al.*, 1972) and Fe-EDTA (Layrisse and Martinez-Torres, 1977) is readily absorbed. Kane and Miller (1984) fractionated protein digests into high and low molecular weight species and measured iron concentrations in the dialysates after dialysis of both fractions against bicarbonate solution. They found that iron concentration in the dialysates of high molecular fraction was very low. These results support the assumption that low molecular weight protein digestion products can enhance iron availability by solubilizing iron and maintaining iron in a low molecular form, while larger peptides or intact proteins may bind iron and inhibit absorption.

2.7.3. Role of amino acids and peptides

Peptides and amino acids released during digestion of different proteins have different affinities for iron (Kane and Miller, 1984). Glutathionine- a tripeptide containing cysteine, was shown to enhance iron absorption (Layrrise *et al.*, 1984). Cysteine alone was only effective when added to a meal after cooking, presumably because it was oxidised during cooking (Martinez-Torres *et al.*, 1981). Amino acids which enhance iron absorption do so by chelating with iron thus preventing its precipitation, or they reduce iron to more available ferrous form.

Maximal protein absorption is further down the small intestine than is iron absorption (Forth and Rummel, 1973). The protein bound iron moves to the intestinal sites beyond the duodenum, which is the site for maximal iron

absorption. If the protein is broken down prior to this than iron is in low molecular from (bound to protein digestion products), hence, can be absorbed by the receptors in the intestine.

2.8. Nutritional relevance of protein-iron interactions

Iron deficiency anaemia is the most common deficiency state in man throughout the world. 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, impair body temperature regulation, impair immune response and lead to a higher incidence of infection (Scrimshaw, 1990).

Iron deficiency can be prevented by ensuring adequate iron intake, either by consuming foods rich in iron, supplementing individuals with medicinal iron, or by fortification of the food supply. Once the prevalence of iron deficiency in a population is known, the dietary intake measured and the foodstuffs likely to influence bioavailability determined, the desired level of iron fortification can be calculated. Though, there are advantages of iron fortification, when supplying iron to large segments of a population there are potential risks. There are four areas of potential concern, increase in infection rate, chronic iron overload, cancer and impaired assimilation of other essential trace elements (Hurrell and Cook, 1990).

As proteins are known to interact with iron in ways which increase or reduce availability. The nature of protein-iron complex formation in foods is one factor which needs consideration when choosing foods to fortify. In general, amino acid-iron complexes are believed to enhance iron availability by chelating with iron thereby forming soluble complexes. Rate of protein digestion and the ability of these digestion products to chelate iron have a significant impact on iron availability.

2.9. Summary

Proteins are able to form complexes with iron and other metal ions. The affinity of different proteins for iron is different due to variation in the amino acid composition.

Milk, soya products, and egg albumen have an inhibitory effect of iron absorption. This effect is due to the ability of the proteins of these foods to bind iron thus making it unavailable. Caseins are the main iron binding proteins in milk; the phosphoserine residues of caseins are responsible for this binding. Soy protein binds significant amount of iron; the phytate and fiber associated with this protein have been implicated for the binding. The low concentrations of crude fiber in soy products indicate that it is not the primary factor. Phytate on its own does not exhibit this inhibitory effect. Phytate forms a stable complex with protein and this complex has the ability to bind iron more tenaciously than phytate or protein alone. Dephytinized soy protein was shown to inhibit iron absorption though it is not as significant as the native protein, indicaing that factors other than phytate are also responsible for the binding. Soy protein is a very large molecule, hence, has the ability to physically entrap iron, thus making it unavailable. This may explain the reduced iron absorption observed in the absence of phytate. Egg albumen has been shown to decrease iron absorption. Conalbumin a protein present in egg albumen has the ability to bind metal ions including iron. But the significant decrease in the absorption of iron in the presence of egg albumen cannot be explained by the binding of iron by conalbumin, the mechanisms or the factors responsible for the reduced iron availability are not fully known.

Chelating agents, such as ascorbic acid and citric acid, have enhancing effect on iron absorption. They have the ability of disrupting the protein iron bond, and form soluble complex with iron thereby making it available. Citric acid can also act as a inhibiting agent under certain conditions like low concentrations of citrate and high concentrations of iron.

Iron availability can be measured by *in vivo* or *in vitro* techniques. *In vitro* studies can be used for screening the factors which influence iron availability and animal and human studies can be carried out to investigate those factors which have a significant effect. The pH, concentration of digestive enzymes and digestion time are the variables which need to be controlled in *in vitro* studies.

Several researchers have worked on protein-iron binding and the results show that proteins have the ability to bind iron and that this complex formation has a significant influence on bioavailability of iron. The affinity of different proteins for iron is different. As the inhibitory or enhancing effect of protein on iron bioavailability depends on the complex formation, it is important to know the binding affinities of commonly consumed food proteins for iron and also the influence of digestion products of these proteins on iron availability. This will help in determining the effects of the protein (inhibition and enhancement) on iron absorption.

OBJECTIVES

1. To investigate the iron binding properties of whey protein, casein, soya protein and egg albumen and determine the binding affinities and number of binding sites for iron.
2. To assess the effects of pH and addition of citric acid and ascorbic acid on the iron binding properties of these proteins.
3. To investigate the release of iron bound to each protein upon digestion of protein by simulating gastrointestinal conditions.
4. To investigate the effect of iron ligands such as citric acid and ascorbic on the release of iron bound to protein.

MATERIALS AND METHODS

4.1. Materials

4.1.1. Iron source

Food grade ferrous sulphate ($FeSO_4$. $7H_2O$), obtained from Riedel-de-haen, Ag Seelze-Hannover, Germany, was the iron source used in the experiments.

4.1.2. Protein sources

Whey protein isolate (WPI) was obtained from Le Sueur Isolates, Minnesota, USA. Sodium caseinate was obtained from the New Zealand Dairy Board, Wellington. Soy protein isolate (SPI) and egg albumen were obtained from the Sigma Chemical Co, St Louis, MO, USA. Each protein (2.5 g) was dissolved in 100 ml of deionized water purified by reverse osmosis followed by treatment with a Milli-Q apparatus (Millipore Corp., Bedford, MA, USA)

4.2. Reagents

All reagents and enzymes were of analytical grade.

4.2.1. Hepes buffer

Hepes buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, obtained from BDH Chemicals, Poole, England), 10 mM, pH 6.6, was prepared by dissolving 2.38 g of Hepes buffer in 800 ml of deionized water, the pH adjusted to 6.6 with 0.1N NaOH, and the volume made upto 1 litre. Sodium chloride (0.4 g) was then added.

4.2.2. Ascorbic acid solution (2.5% w/v)

2.5 g of ascorbic acid (obtained from Sigma Chemical Co, St Louis, MO, USA) was dissolved in 100 ml Hepes buffer.

4.2.3. Citric acid solution (2.5% w/v)

2.5 g of citric acid (obtained from BDH Chemicals, Poole, England) was dissolved in 100 ml Hepes buffer.

4.2.4. Enzyme solutions

Pepsin (16.0 g) was dissolved in a small volume of 0.1 M HCl, and brought to 100 ml with 0.1 M HCl. Pancreatin (400 mg) and bile extract (2.5 g) were dispersed in 1 litre of 0.1 M sodium bicarbonate. Both pepsin (frog; activity 1 Anson unit per gram) and Pancreatin-bile extract (porcine; activity equivalent to 3 x USP) were obtained from Sigma Chemical Co, St Louis, MO, USA.

4.3. Analytical Methods

4.3.1. Iron concentration

Iron concentration in the samples was determined by atomic absorption spectroscopy (Varian AA 175-series, Varian Techtron Ltd, Springvale, Australia). Iron standards were prepared by using a certified iron absorption reference solution obtained from BDH Limited, Poole, England.

4.3.1.1. Acid digestion procedure

20 ml of HCl solution (500 ml of HCl and 220 ml of deionized water) was added to 20 ml of the sample. This mixture was boiled for five minutes and filtered through Whatman filter paper (No 1). The filtrate was diluted with deionized water to make the final volume to 20 ml and then analyzed for iron.

4.3.2. Protein concentration

The protein concentration was estimated by determining the total nitrogen by Kjeldahl method, using Kjeltec 1026 system (Tecator, Sweden). Protein contents were calculated from the nitrogen percentage by multiplying by an empirical factor. The factor used for casein and whey protein was 6.38 and that for soy protein and egg albumen was 6.25.

4.4. Experimental procedures

4.4.1. Solubility of ferrous sulphate

Solubility tests were done to determine the effects of pH and iron concentration on the solubility of ferrous sulphate and thus decide the experimental conditions for carrying out the binding studies. Solubility of ferrous sulphate was determined by dissolving the equivalent of 0.1-0.5 mg/ml iron in 50 ml Hepes buffer and adjusting the pH of the contents each flask to 4, 5, 6, or 7 with 0.5 M HCl or 0.5 M NaOH. After thorough mixing, the flask contents were centrifuged for 20 minutes at 10,800 *g* and the supernatants collected. The flasks were then washed and any remaining residues were resuspended in 50 ml deionized water, centrifuged, and the washings from three such treatments added to the original supernatants. Iron concentrations in the supernatants were determined using atomic absorption spectrophotometry. This procedure was repeated three times at each pH and concentration. Under these conditions ferrous sulphate was found to be >91% soluble at all concentrations and pHs, except at pH 7. At pH 7 solubility decreased from 88% to 81% as the concentration was raised from 0.1 mg to 0.5 mg per ml.

4.4.2. Iron binding studies

Binding of iron to WPI, sodium caseinate, SPI and egg albumen was studied by dissolving ferrous sulphate containing an equivalent of 2.5 to 375 mg iron in 40 ml Hepes buffer. To this solution, 10 ml of protein solution was added to give a final concentration of 5 mg/ml protein and 0.01 to 1 mg/ml iron. The mixture was incubated for an hour at room temperature (15°C - 18°C) and then centrifuged at 10,800 *g* for 20 minutes. After centrifugation, the supernatant was decanted from the sediment fraction and aliquots of the supernatant analyzed for iron and protein concentrations. To determine iron binding to soluble proteins, the supernatant was ultrafiltered using an Amicon stirred ultrafiltration cell (Model 8050, Amicon Div., W.R. Grace and Co. Denvers, MA, USA) at a pressure of 300 kPa with a Diafilo UF membrane YM10 (MW cut off 10,000). The permeate obtained was analyzed for

concentration of iron.

4.4.3. Effect of pH on iron binding

The pH of the protein-iron mixtures was adjusted over the range 2 - 7 with 0.5 M HCl or 0.5 M NaOH prior to incubation; the same procedure as in binding studies was carried out.

4.4.4. Effect of chelating agents on iron binding

After incubating the protein-iron mixture for 1 hour, as in binding studies, ascorbic acid or citric acid stock solution was added to the samples so as to provide 25 - 250 mg of acid. The samples were then incubated for a further 30 minutes, centrifuged, ultrafiltered and the supernatant and the permeate analyzed for iron concentration.

4.4.5. In vitro availability studies

4.4.5.1. Protein iron mixtures

5 g protein and ferrous sulphate, to an equivalent of 100 mg iron, were dissolved in 250 ml Hepes buffer (pH 6.5). The mixture was incubated for one hour and then freeze dried. These mixtures were used for the availability studies.

Preliminary trials were carried out in order to choose the method that would give the reliable results under the experimental conditions. The procedure outlined below was followed in the first experiment. It is the method suggested by Miller and Schricker (1981) which resembles several published *in vitro* methods (Jacobs and Greenman, 1969; Narasinga Rao and Prabhavathi, 1978)

4.4.5.2 Pepsin digestion

400 mg of freeze dried protein-iron mixture was taken in two flasks, dissolved in 40 ml of 0.1 M HCl and pH was adjusted to 2.0. To the contents of one flask, pepsin stock solution was added in an amount that provided 0.5 g

pepsin per 100 g protein-iron mixture, the contents of another flask (without pepsin) acted as control. The samples (protein-iron mixture with and without enzyme) were incubated at 37°C for 2h in a shaking water bath. After incubation, a 20 ml aliquot of each (control and pepsin digest) was used to measure titratable acid (number of equivalents of KOH required to titrate the combined pepsin digest pancreatin-bile extract mixture to pH 7.5; 0.5 M KOH was used in the titration) and free iron concentration.

4.4.5.3. Pancreatin digestion

Segments of dialysis tubing containing 25 ml water and an amount of sodium bicarbonate, equivalent to the titratable acid measured previously, were placed in a beaker containing a 20 ml aliquot of pepsin digest or control sample from the above experiment. Sodium bicarbonate neutralizes the pepsin digest. The use of bicarbonate in dialysis tubing is to allow for a slow increase in the pH before and during pancreatin digestion. This closely parallels the events occurring when food leaves the stomach and enters the duodenum. The beakers were sealed with parafilm and incubated at 37°C in a shaking water bath until the pH was raised from 2 to about 5 (approximately 30 minutes). Pancreatin bile extract mixture (1.0 ml) was added to each beaker and the incubation was continued for another 2 h. At the end of the incubation period, the dialysis tubes were removed, rinsed with distilled water, and the contents (dialysates) weighed and analyzed for the amount of iron present. The iron present in the dialysate is low molecular weight iron (free, or bound to low molecular weight protein digestion products), hence, available.

It was observed that the concentration of iron in the dialysates was too low to be measured accurately, suggesting that the incubation time used might not be sufficient to complete the enzymatic digestion of protein and thus the release of iron bound to protein.

The above procedure was repeated with the following changes:
(i) the pH adjustment before adding pancreatin was direct, i.e, by addition of 0.5 M HCl or 0.5 M NaOH,
(ii) the pancreatin digestion time was increased from 2h to 4h and
(iii) separation of protein bound iron and free iron was performed using ultrafiltration, with a membrane with a molecular with MW cut off 10,000.

This modified procedure gave satisfactory and reproducible results, the concentration of free iron was higher than obtained by the previous procedure.

4.4.5.4. Modified procedure for iron availability studies

The final method for iron availability tests was designed by taking into consideration the above mentioned factors.

Pepsin digestion

400 mg of protein-iron mixture was suspended in 40 ml 0.1 M HCl. The pH of the mixture was adjusted to 1.6 with 0.5 M HCl or 0.5 M NaOH. The soy protein-iron mixture was not soluble in 0.1 M HCl so it was dissolved in deionized water and then the pH was adjusted to 1.6 with 0.5 M HCl. To a beaker, pepsin stock solution was added in an amount that provided 0.5 g pepsin per 100 g of protein-iron mixture. The samples were incubated at 37°C in a shaking water bath for 2 h. Aliquots (20 ml) of pepsin digest and control (no enzyme) were ultrafiltered; the permeates digested with HCl and analyzed for the concentration of iron.

Pancreatin digestion

The pH of the pepsin digest and control was adjusted to 7 with 0.5 M NaOH. Pancreatin bile extract mixture (1 ml of the stock solution) was added to the pepsin digest. The samples were incubated at 37°C for 4h in a shaking water bath. Aliquots of pancreatin digest and control (no enzyme) were ultrafiltered; the permeates digested with HCl and analyzed for iron

concentration.

4.4.5.5. Effect of chelating agents on iron release

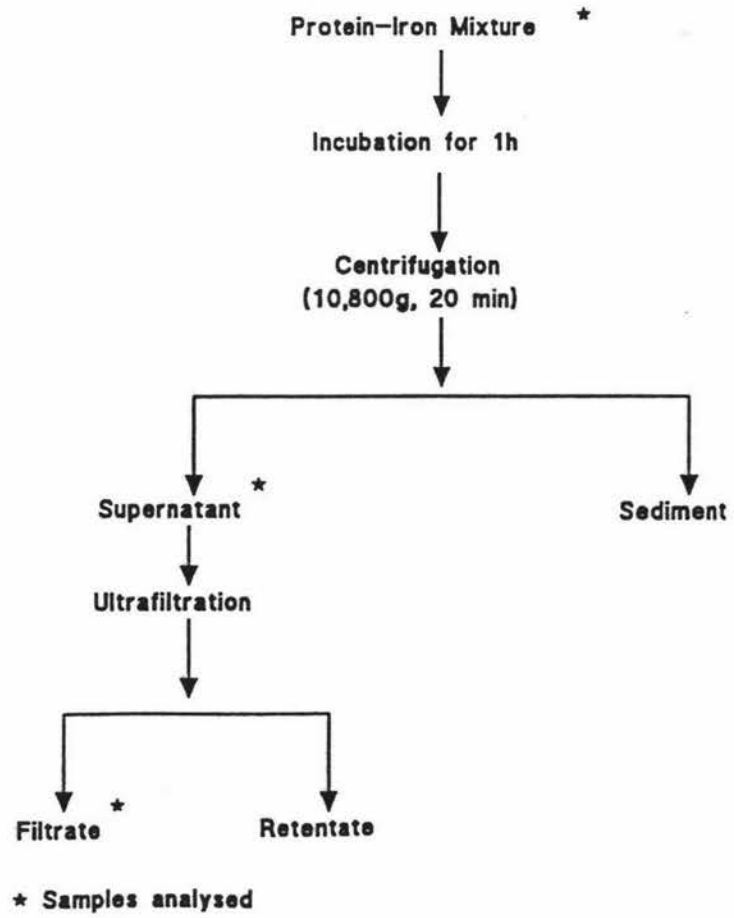
100 mg of ascorbic acid or citric acid was added to the protein-iron mixture and dissolved in HCl prior to enzyme addition, and a similar procedure as the availability studies was carried out.

BINDING OF IRON TO WHEY PROTEIN, CASEIN, SOYA PROTEIN AND EGG ALBUMEN

5.1. Experimental Design

The method used was adapted from Nelson and Potter (1979) for determining the binding of iron to proteins. The general procedure is outlined in Figure 5.1. Protein-iron mixtures were centrifuged at 10,800 *g* for 20 mins and the supernatants obtained were analyzed for iron and protein concentrations. The supernatants were ultrafiltered and the permeates analyzed for iron concentration. Binding of iron to soluble and insoluble fraction was calculated as follows:

Insoluble protein = Total protein - protein in the supernatant
Iron bound to insoluble protein = Total iron - iron in the supernatant
Iron bound to soluble protein = Iron in the supernatant - iron in the permeate



5.2. Effect of iron concentration on protein solubility

Figure 5.2.a-d shows the effect of ferrous iron on the solubility (measured as the quantity of protein sedimentable at 10,800 *g* for 20 min) of whey protein isolate (WPI), casein, soya protein isolate (SPI) and albumen. All proteins showed a marked increase in the amount of insoluble protein with increase in iron concentration from 0 to ~ 3 mM. Addition of iron at > ~ 3 mM had only a small effect on the amounts of insoluble protein. WPI showed the greatest increase in the insoluble protein followed by SPI, albumen and casein.

The decrease in protein solubility on addition of iron observed in the present study agrees with the findings of Nelson and Potter (1979) and Satterlee and Schnepf (1984). The positive charge on iron may be responsible for holding some of the protein molecules together by forming ionic linkages between negative charges on the protein molecules (Satterlee and Schnepf, 1984), thereby increasing the amount of sedimentable protein.

The amount of iron co-sedimented with the protein increased with increase in iron concentration but the extent of increase was dependent on the type of the protein present (Fig. 5.2a-d). The concentration of iron in the sediment increased from 0.045 to 0.147 mM for WPI, 0.05 to 1.89 mM for casein, 0.06 to 1.3 mM for SPI and 0.14 to 3.05 mM for albumen when the concentration of total added iron was increased from 0.179 to 17.9 mM. In the absence of the protein and under similar experimental conditions, ~ 97% of added iron, (as ferrous sulphate) was soluble. Therefore, iron sedimented with the protein on centrifugation could be taken as iron bound to the insoluble protein fraction.

5.3. Binding of iron to insoluble protein fraction

In all proteins studied, a substantial amount of iron was bound to the insoluble fraction (Fig. 5.3a-d). In the case of casein and albumen, (Fig. 5.3 a,b) the amount of iron bound/g insoluble protein increased with increase

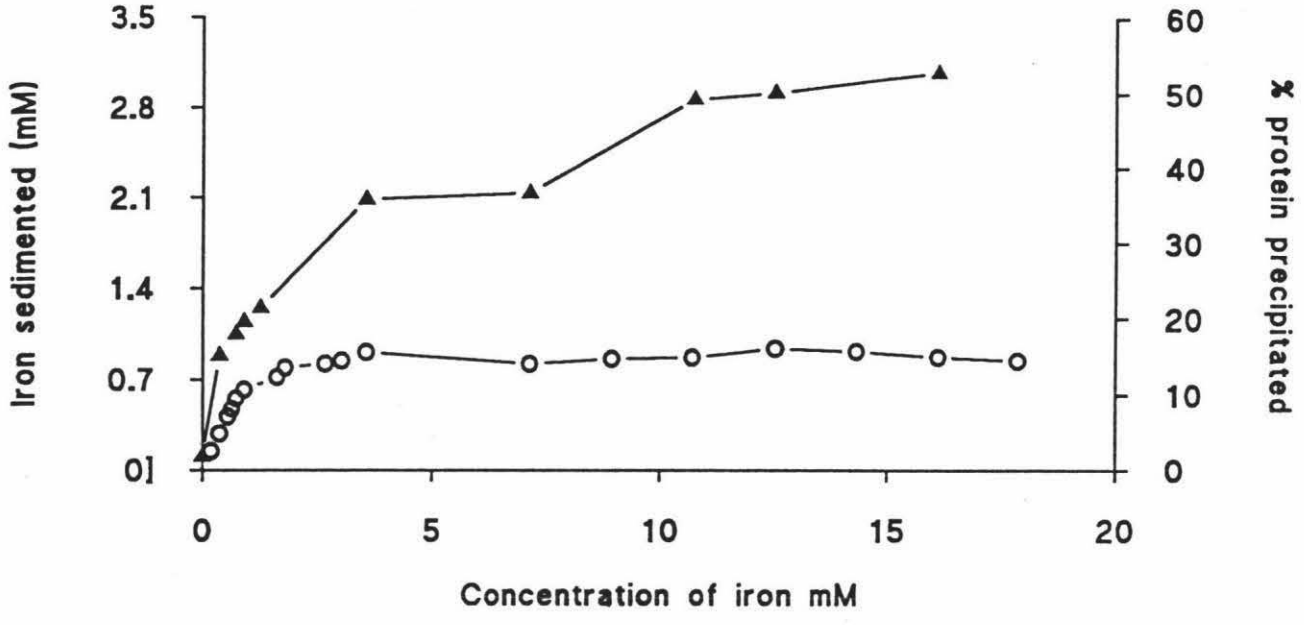


Fig 5.2 (a). Influence of iron concentration on the solubility of WPI (▲) and iron (o) in 10 mM Hepes buffer at pH 6.6.

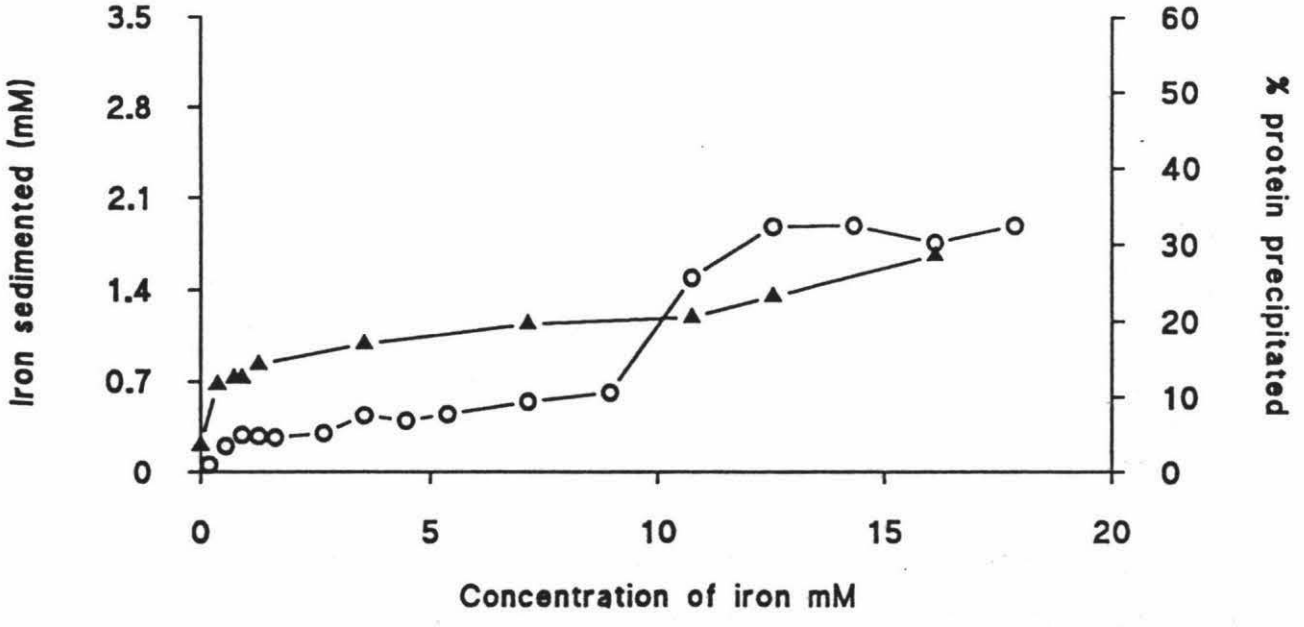


Fig 5.2 (b). Influence of iron concentration on the solubility of Casein (▲) and iron (o) in 10 mM Hepes buffer at pH 6.6.

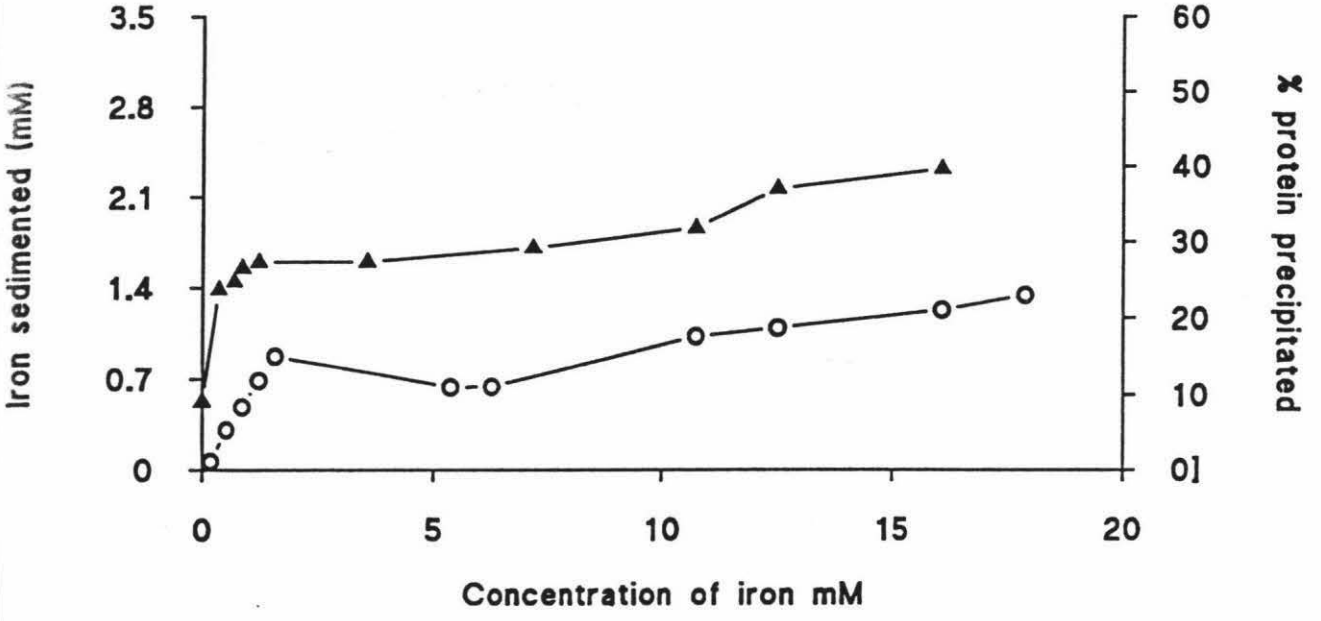


Fig 5.2 (c). Influence of iron concentration on the solubility of SPI (▲) and iron (o) in 10 mM Hepes buffer at pH 6.6.

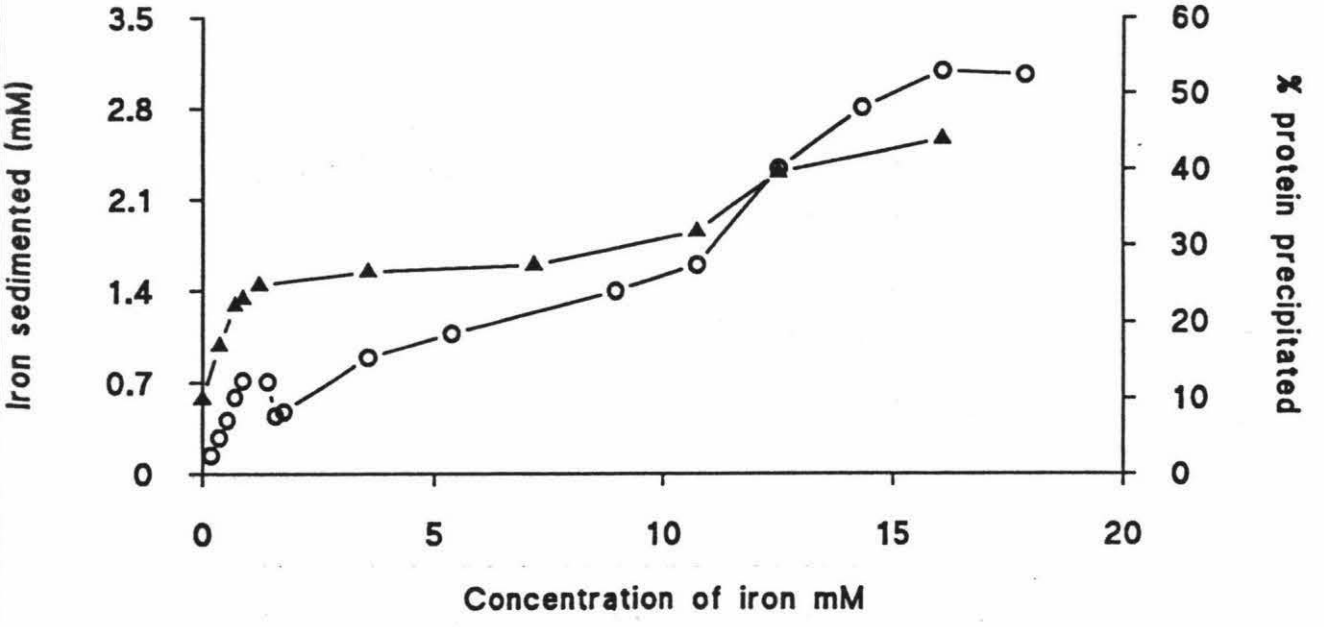


Fig 5.2 (d). Influence of iron concentration on the solubility of Albumen (▲) and iron (o) in 10 mM Hepes buffer at pH 6.6.

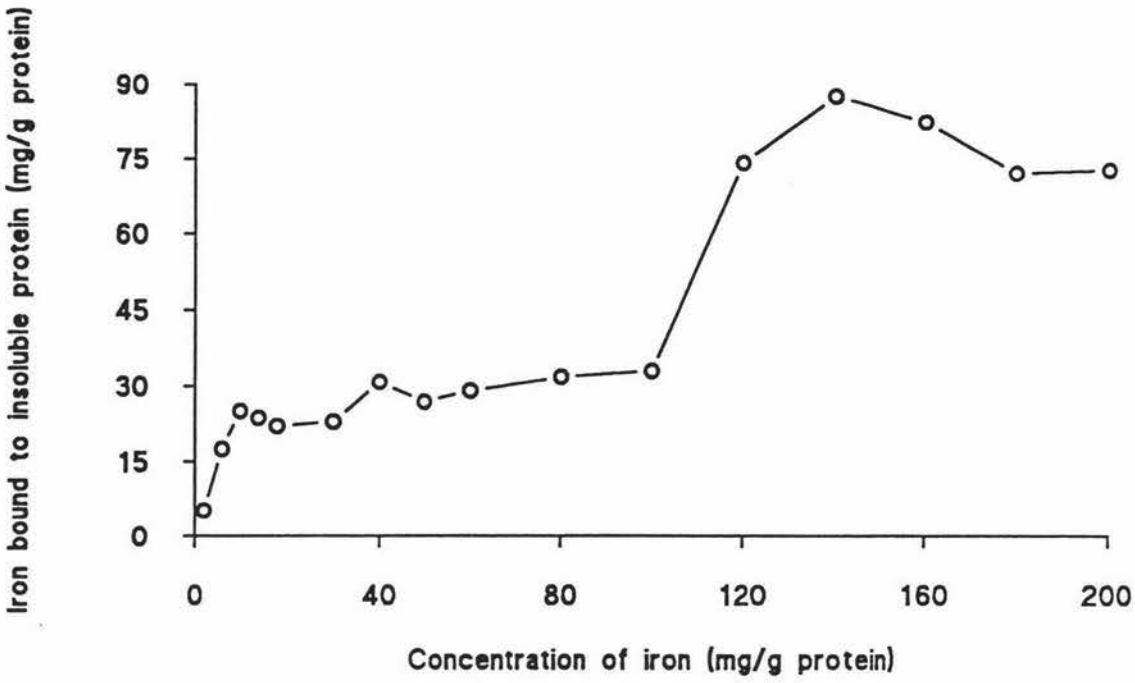


Fig 5.3 (a). Influence of iron concentration on the binding of iron to the insoluble fraction of casein in 10 mM Hepes buffer at pH 6.6.

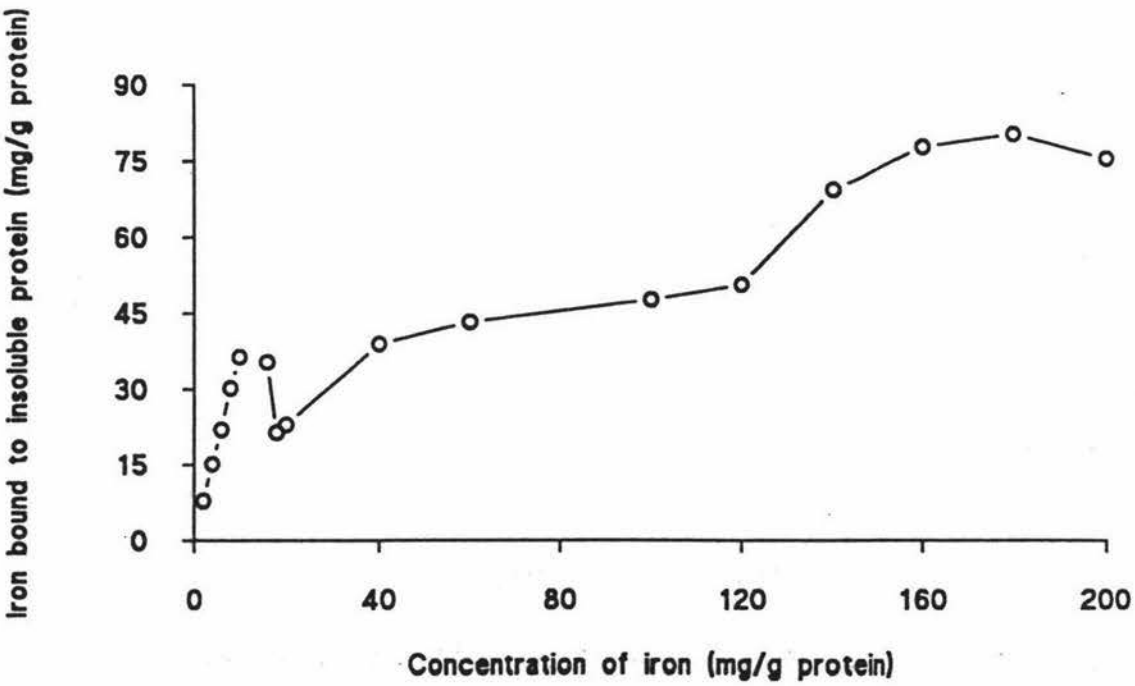


Fig 5.3 (b). Influence of iron concentration on the binding of iron to the insoluble fraction of egg albumen in 10 mM Hepes buffer at pH 6.6.

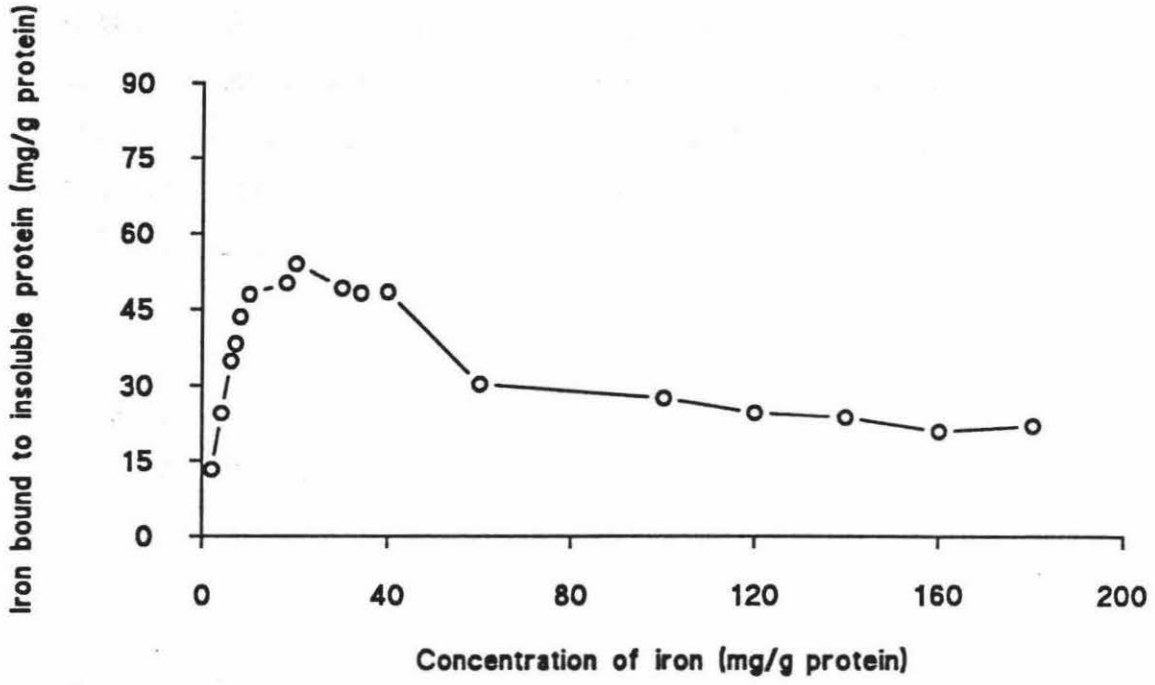


Fig 5.3 (c). Influence of iron concentration on the bidning of iron to the insoluble fraction of WPI, in 10 mM Hepes buffer at pH 6.6

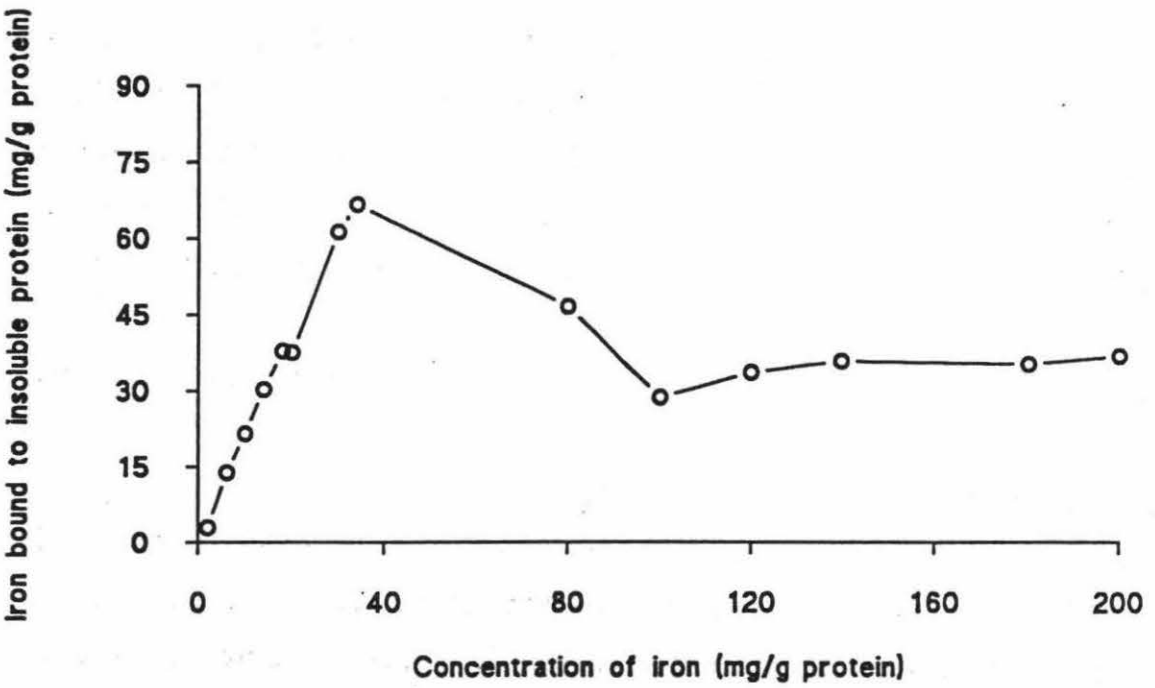


Fig 5.3 (d). Influence of iron concentration on the binding of iron to the insoluble fraction of SPI in 10 mM Hepes buffer at pH 6.6

in iron concentration and there appeared to be two stages of binding. There was a steady increase in the amount of iron bound to the insoluble fraction as the concentration of added iron was increased from 0 to 20 mg/g protein. Between iron concentrations of 20-120 mg/g protein, no significant changes in binding occurred. However, higher concentrations of iron led to a significant increase in bound iron and finally the binding isotherm levelled off at ~ 150mg iron/g protein.

WPI and SPI showed different patterns of binding (Fig. 5.3c,d). The amount of iron bound to the insoluble fraction increased as the iron concentration was increased from 2 mg to 40 mg/g protein, but above this concentration, there was a decrease in binding.

Thus, the amount of iron added to achieve maximum binding varied for each protein; these amounts were 40 mg for WPI and SPI, 160 mg for casein and 180 mg for albumen. The amount of iron bound/g of the insoluble fraction was highest for casein (87 mg) followed by albumen (80 mg), SPI (66 mg) and WPI (53 mg).

Nelson and Potter (1979) reported that at 20 mg added iron/g protein, the insoluble fractions of SPI, casein and albumen bound 16.7 mg, 8.7 mg and 6.3 mg iron, respectively. In the present study, at similar iron/protein ratios, the insoluble fractions of SPI, casein and albumen bound 9.6 mg, 3.7 mg and 5.2 mg iron, respectively. The observed differences between the two studies may be due to higher centrifugation speed used by Nelson and Potter (1979) (12,000 *g vs* 10,800 *g* in the present study) to separate the insoluble fractions.

5.4. Binding of iron to soluble protein fraction

The isotherms for binding of iron to the soluble fractions of WPI, casein, SPI and albumen as a function of iron concentration are shown in Fig. 5.4 (a,b,c,d). As the total iron concentration in the protein solutions increased, the binding of iron to all proteins increased until it approached a final

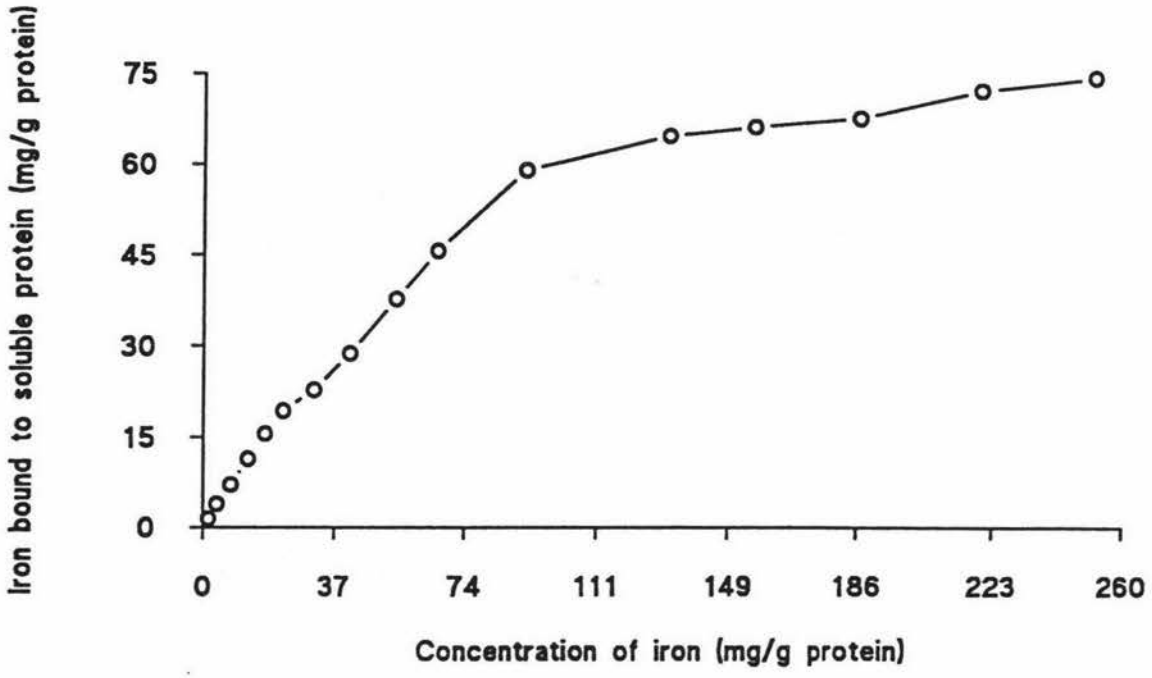


Fig 5.4 (a). Influence of iron concentration on the binding of iron to the soluble fraction of casein in 10 mM Hepes buffer at pH 6.6.

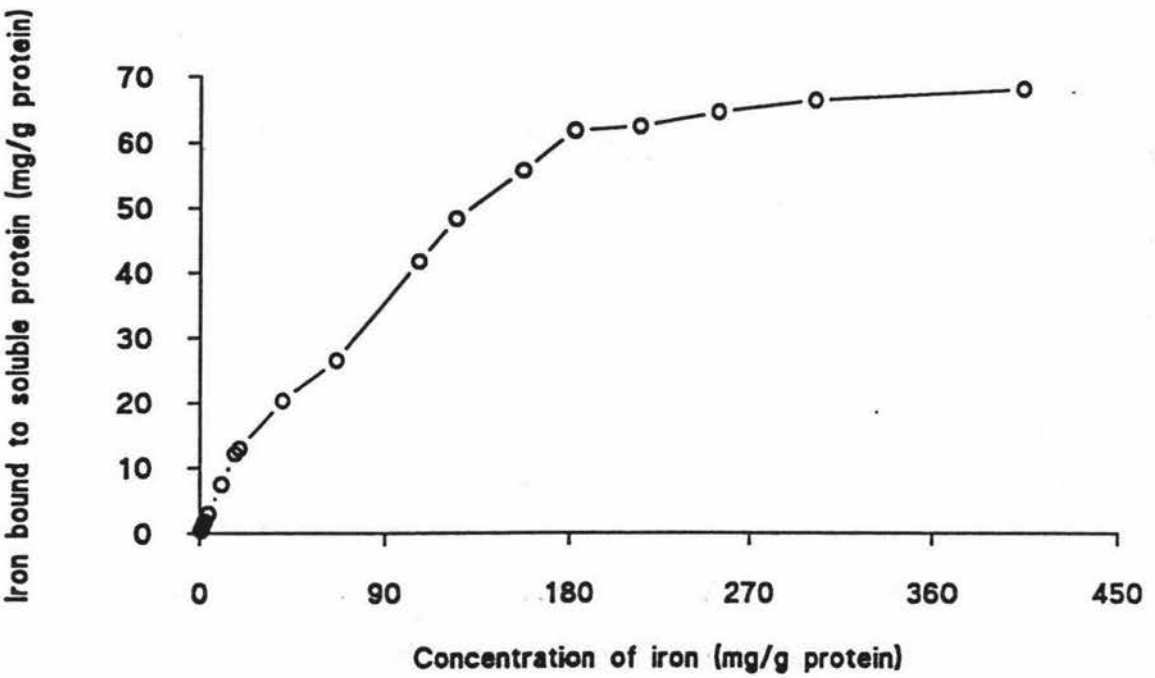


Fig 5.4 (b). Influence of iron concentration on the binding of iron to the soluble fraction of egg albumen in 10 mM Hepes buffer at pH 6.6.

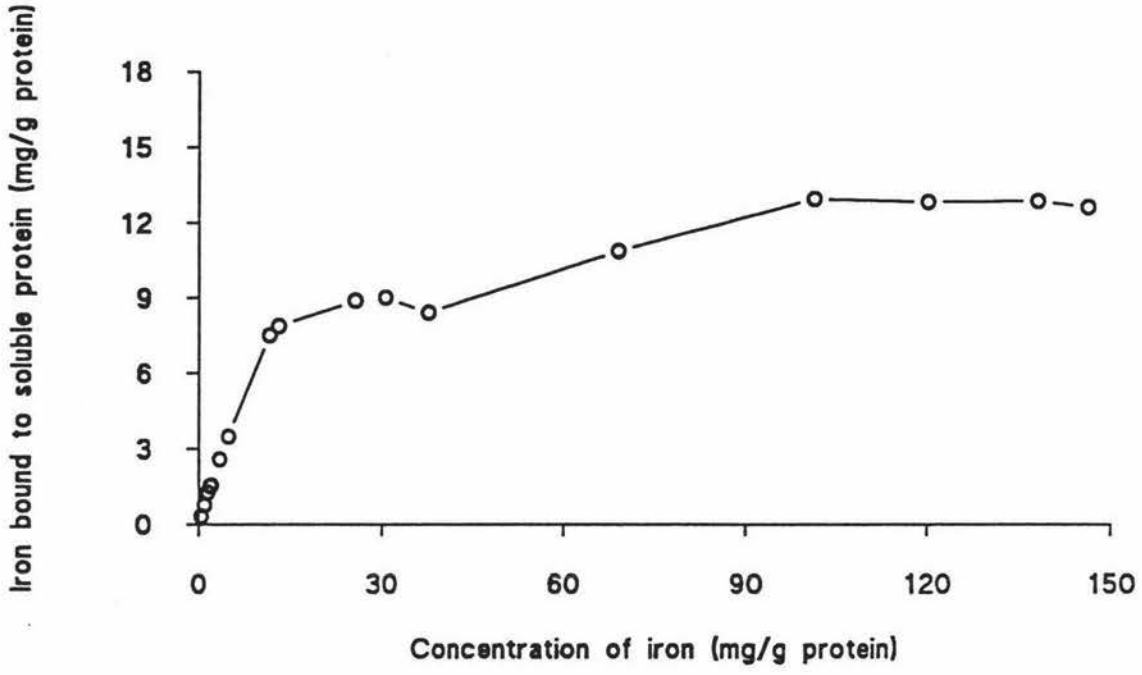


Fig 5.4 (c). Influence of iron concentration on the binding of iron to the soluble fraction of WPI in 10 mM Hepes buffer at pH 6.6.

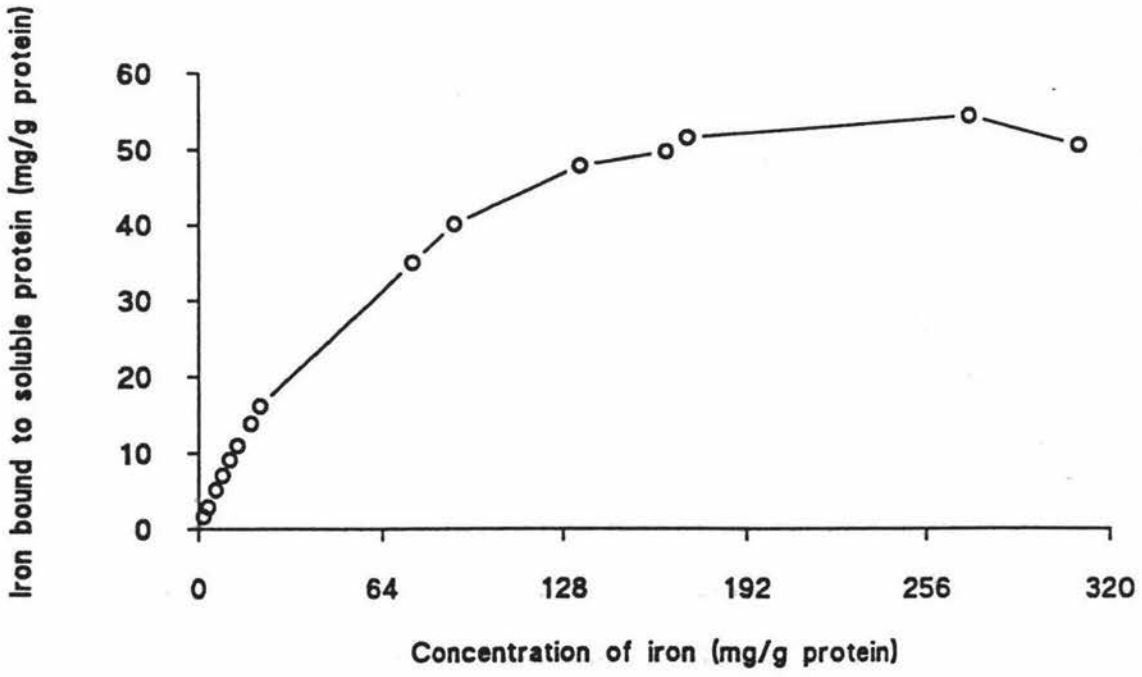


Fig 5.4 (d). Influence of iron concentration on the binding of iron to the soluble fraction of SPI in 10 mM Hepes buffer at pH 6.6.

plateau. The final plateau values in the curves differed among different proteins, which indicated that different iron concentrations were required by each protein to achieve maximum binding. The binding isotherms indicated that casein bound maximum quantities of iron and approached saturation at 74 mg bound iron/g protein, followed by albumen at 68 mg and SPI at 54 mg. WPI bound much less iron than casein, SPI or albumen and saturation was approached at ~12 mg of iron bound/g protein. When expressed in terms of moles of iron bound per mole of protein (calculated using molecular weights of 18,000, 22,000, 350,000 and 68,000 for WPI, casein, SPI and albumen, respectively) the trend was different. One mole of SPI was observed to bind 344 moles of iron, followed by albumen which bound 80 moles of iron; Casein bound 28 moles and WPI bound 6 moles of iron.

Studies by Basch *et al.* (1973) and Demott and Dincer (1976) on the distribution of added iron in milk fractions showed that the whey protein fraction bound less iron than casein fraction. Demott and Dincer (1976) found that whey fraction bound 9% of the added iron as compared to 85% bound by the casein fraction. Similar values were also obtained by Basch *et al.* (1973). The values obtained in the present study show that whey proteins bind less iron than casein; WPI bound 12 mg iron/g protein and casein bound 74 mg iron. Singh *et al.* (1988) showed that the major whey proteins had little capacity for zinc binding compared with caseins. Similar observation was also made with regards to calcium binding capacities of various milk fractions (Dickson and Perkins, 1971).

Satterlee and Schnepf (1984) found that SPI bound 19.6 mg iron/g protein, but in the present study, at similar protein/iron ratios, SPI bound 30 mg iron. However, these workers used different conditions (pH and buffer) than those used in the present study. Studies by Appu Rao and Narsinga Rao (1976) showed that soya proteins bound significant amounts of zinc, calcium and magnesium.

5.4.1. Binding sites and binding constants

The binding data were analysed using the Scatchard equation (Scatchard, 1949) to evaluate the binding constants (k), and the maximum number of binding sites (n).

The equation used was

$$v/A = k(n-v)$$

where v is the number of moles of iron bound to a mole of protein, A is the number of moles of free iron. The equilibrium binding constant (k) is given by the slope of the Scatchard plot *i.e.* v/A *vs* v. The equilibrium binding constant is a measure of binding strength (Kroll, 1984) and it gives an indication of the affinity of iron (ions) for the binding sites on the protein molecule. The maximum number of binding sites (n) is given as the intercept of the plot on the abscissa.

Figure 5.5 (a,b,c,d) shows the Scatchard plots of all four proteins. These plots could be fitted to two straight lines which indicates that the binding sites are non-equivalent (Rao and Rao 1976). The initial linear portion gave, on extrapolation to x-axis, a value of n. The values of n for WPI, casein, SPI and albumen were 1.7, 13.6, 201 and 42.01 respectively. The extrapolation of the second linear portion gave n values of 7.02, 37.7, 121.6, and 426 for WPI, casein, SPI and albumen, respectively. The binding constants obtained for the binding sites were 11.2, 4.76, 2.95, 1.37 for WPI, casein, SPI and albumen, respectively.

The plots indicate that there are two types of binding sites for iron on these proteins and that the binding sites are non-equivalent; the first few ions were bound more strongly than the remaining.

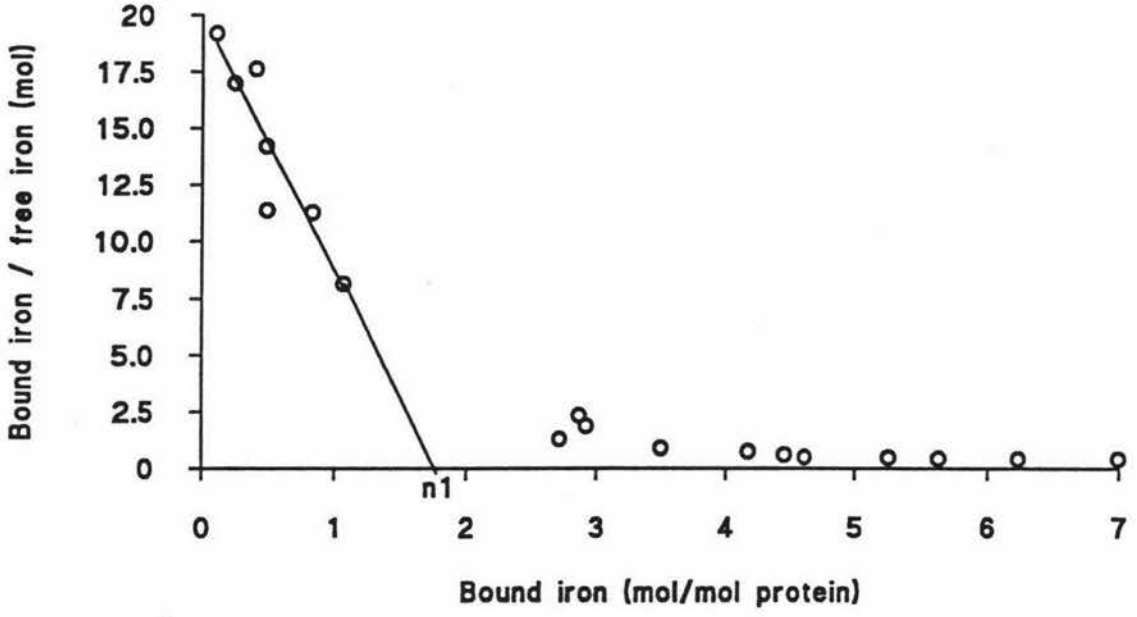


Fig 5.5 (a). Scatchard plot for the binding of iron to WPI

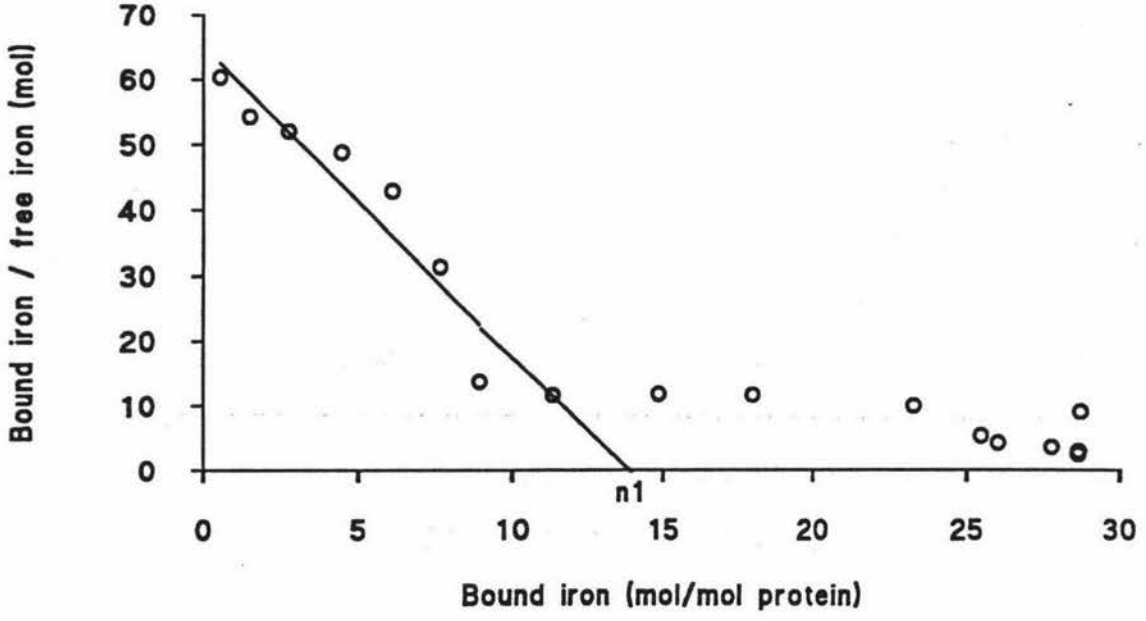


Fig 5.5 (b). Scatchard plot for the binding of iron to casein.

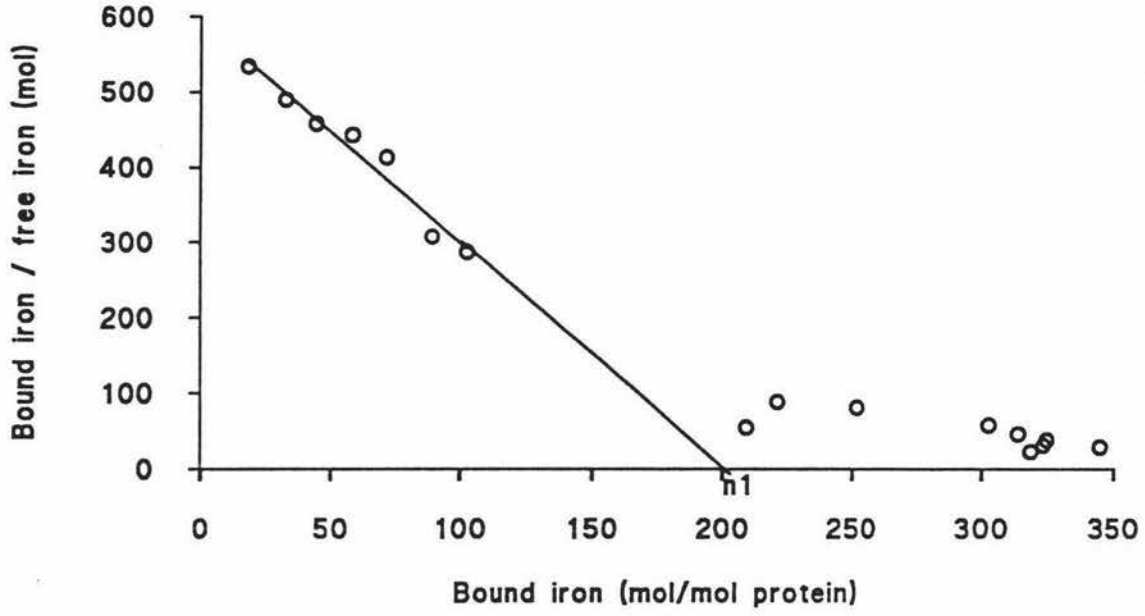


Fig 5.5 (c). Scatchard plot for the binding of iron to SPI

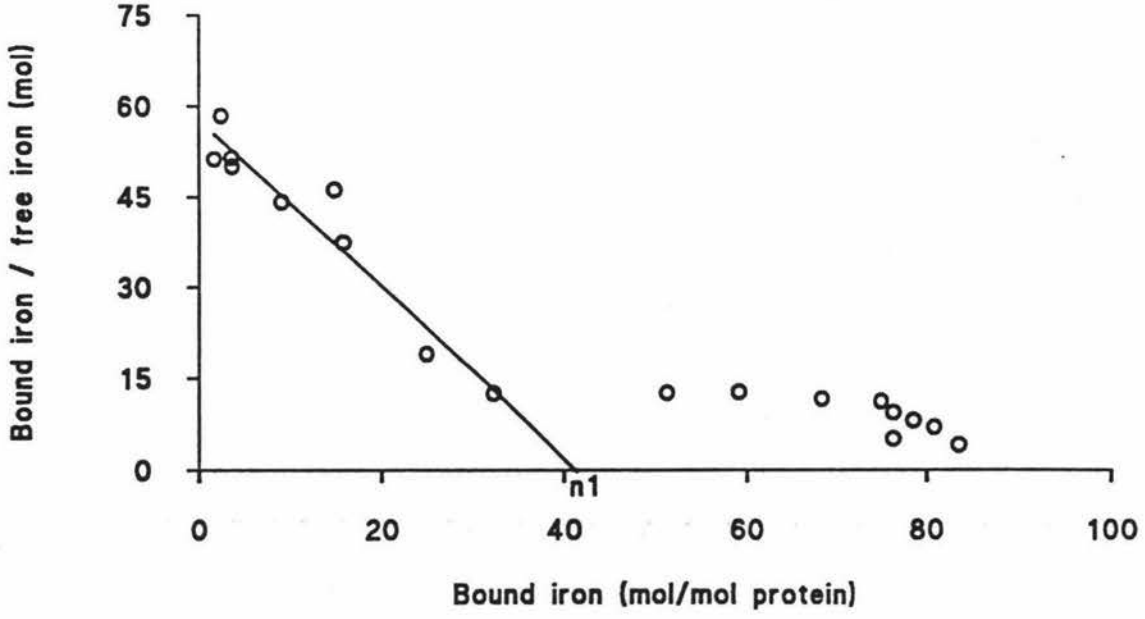


Fig 5.5 (d). Scatchard plot for the binding of iron to Egg albumen.

Table 5.1: Binding constants and maximum number of binding sites

Protein	Binding constant ($k\ mol^{-1}$)	Max binding sites (n)
WPI	11.22	1.78
Casein	4.76	13.6
SPI	2.95	201
Albumen	1.37	42.01

The data shows that WPI has ~2 binding sites for iron; the binding constant value obtained (11.22) suggests that the binding was very strong. No data is available in the literature on the number of binding sites for iron on WPI. Singh *et al.* (1988) found two distinct classes of binding sites for zinc on whey proteins, and the total number of binding sites of both classes was ~2 each for both α-lactalbumin and β-lactoglobulin.

Casein was found to have 13 binding sites for iron, which are greater than the binding sites reported for zinc (6) (Singh *et al.*, 1988). Phosphoserine residues of the caseins have been shown to be the primary binding sites for calcium, zinc, magnesium, copper and iron (Dickson and Perkins, 1971; Hegenauer, 1979; Lonnerdal *et al.*, 1982; Singh *et al.*, 1988). However, since the total number of binding sites (n=13) exceeds the number of phosphoserine residues (~8), it appears that caseins contain iron binding sites other than phosphoserine residues. Histidine and carboxylic groups which have been implicated as possible binding sites for zinc in caseins (Singh *et al.*, 1988) may have iron binding capacity as well.

SPI was found to have ~200 binding sites for iron, the value obtained for binding constant (2.95) indicates that the binding was not as strong as binding by other proteins. Earlier researchers have reported 24-43 binding

sites for calcium, 16 binding sites for magnesium and 18 binding sites for zinc on soya proteins (Saio *et al.*, 1968; Rao and Rao, 1976; Kroll, 1984). The higher value obtained in the present study for iron binding sites indicate that soya protein bind significantly more iron when compared to other metal ions. Histidine has been implicated for the metal ion binding properties of soya proteins (Appu Rao and Narsinga Rao, 1976; Kroll, 1984). Norsworthy and Caldwell (1987) found 330 binding sites for zinc on soya bean glycinin (11S fraction) and suggested that as this was much higher than the number of histidine residues (~18); carboxyl groups were suggested to be the most likely binding sites. Amino acid analysis has indicated a total of 455 acidic groups. The side chain carboxyl groups of aspartic and glutamic acid have been implicated for calcium binding ability of soya proteins (Kroll, 1984). The carboxyl groups along with histidine may account for the large number of binding sites (200) obtained for iron in the present study.

The maximum number of binding sites for iron on albumen were 42.01 and the binding constant value of obtained was 1.37. Conalbumin present in egg albumen has been shown to bind di-valent and tri-valent metal ions including iron (Warner and Weber, 1953). The affinity of albumen for iron observed in the present study can be attributed to the presence of conalbumin.

5.5. Effect of pH on the solubility of protein and iron

Iron-binding isotherms for WPI, casein, SPI and albumen indicated that the maximum binding was approached at 100 mg added iron/g WPI, 160 mg/g casein and SPI, and 180 mg iron/g albumen. Therefore, the effects of pH on binding were carried out at these concentrations of iron.

Figure 5.6 (a,b,c,d) shows the solubilities of WPI, casein, SPI and albumen at different pH values with or without added iron. All proteins exhibited distinct protein solubility curves over the pH range studied and in general addition of iron decreased the solubility of all proteins. Changes in protein

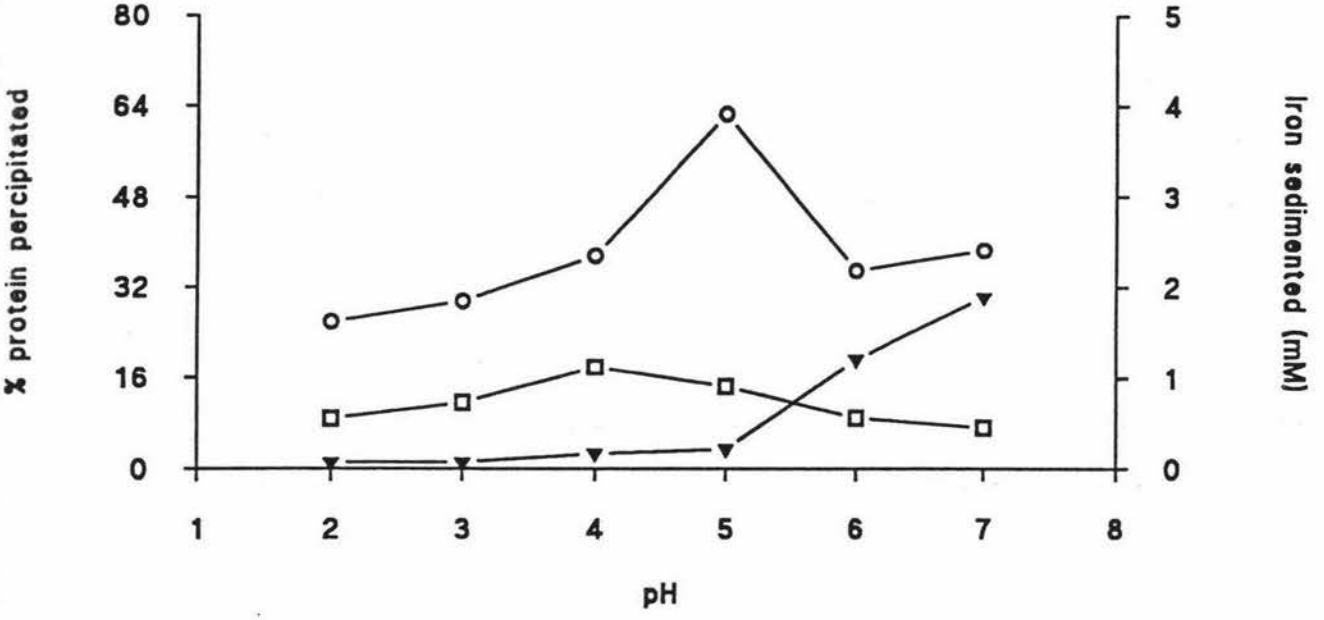


Fig 5.6. (a) Effect of pH on the solubility of WPI and iron at protein : iron ratio of 1 : 0.1. (o) protein with iron, (□) protein without iron and (▼) iron

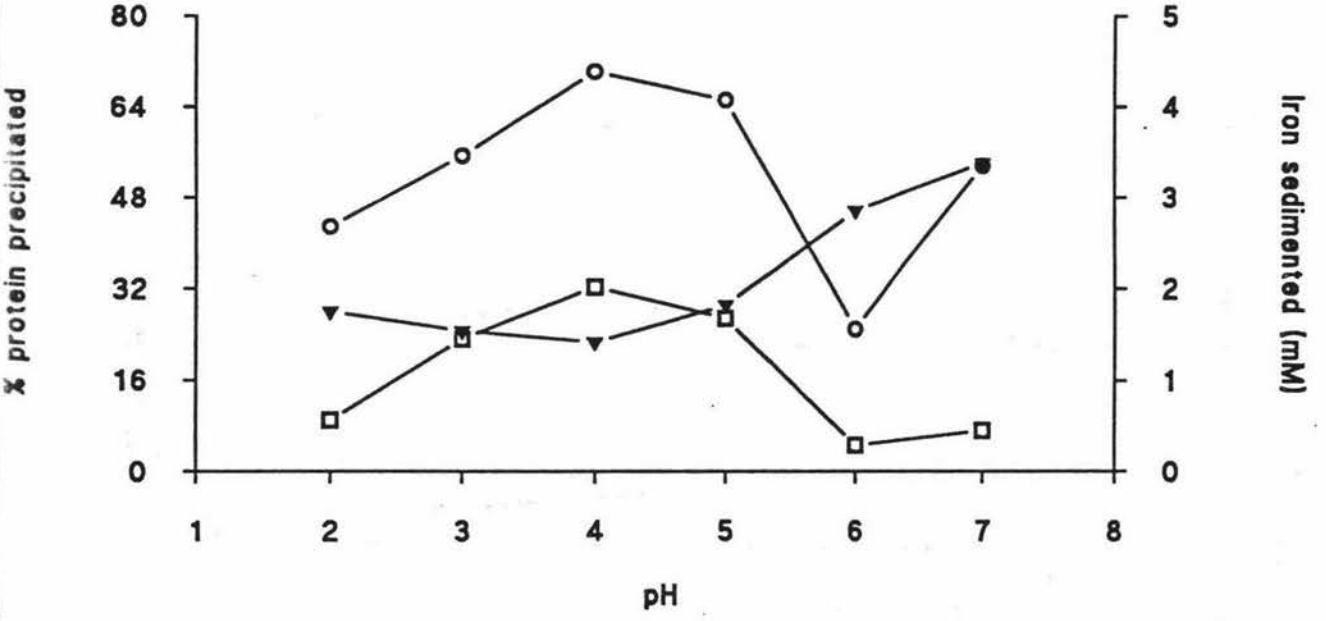


Fig 5.6 (b) Effect of pH on the solubility of casein and iron at protein : iron ratio of 1 : 0.16. (o) protein with iron, (□) protein without iron and (▼) iron

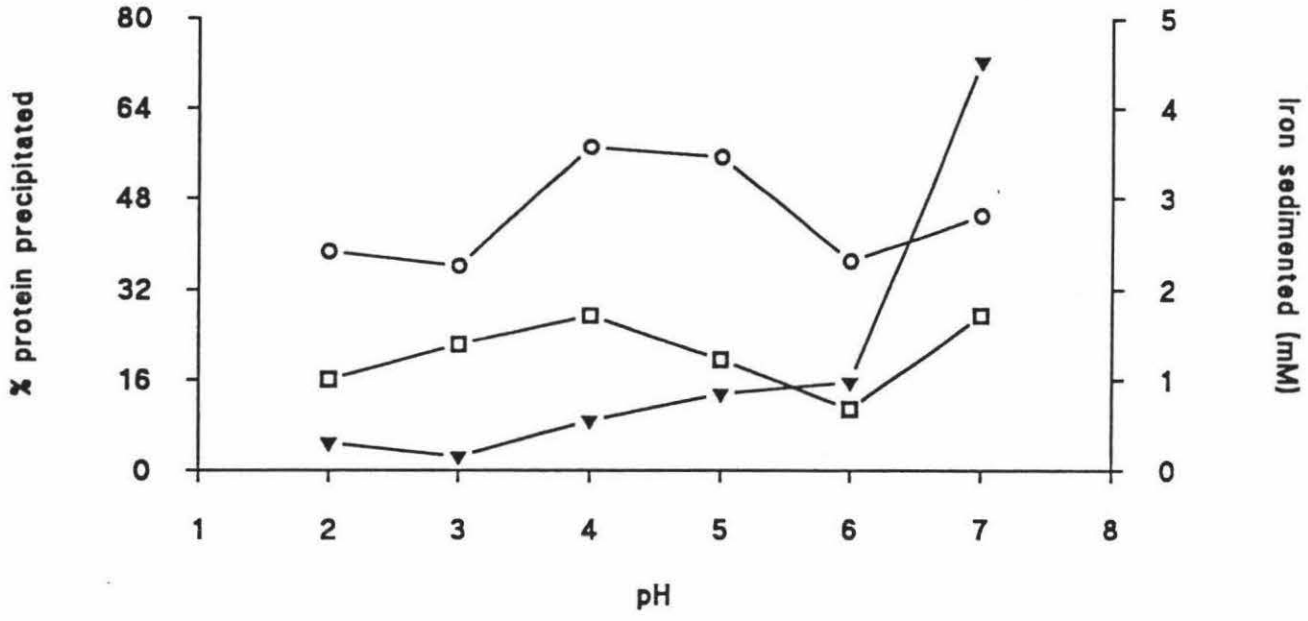


Fig 5.6 (c) Effect of pH on the solubility of SPI and iron at protein : iron ratio of 1 : 0.16. (o) protein with iron, (□) protein without iron and (▾) iron

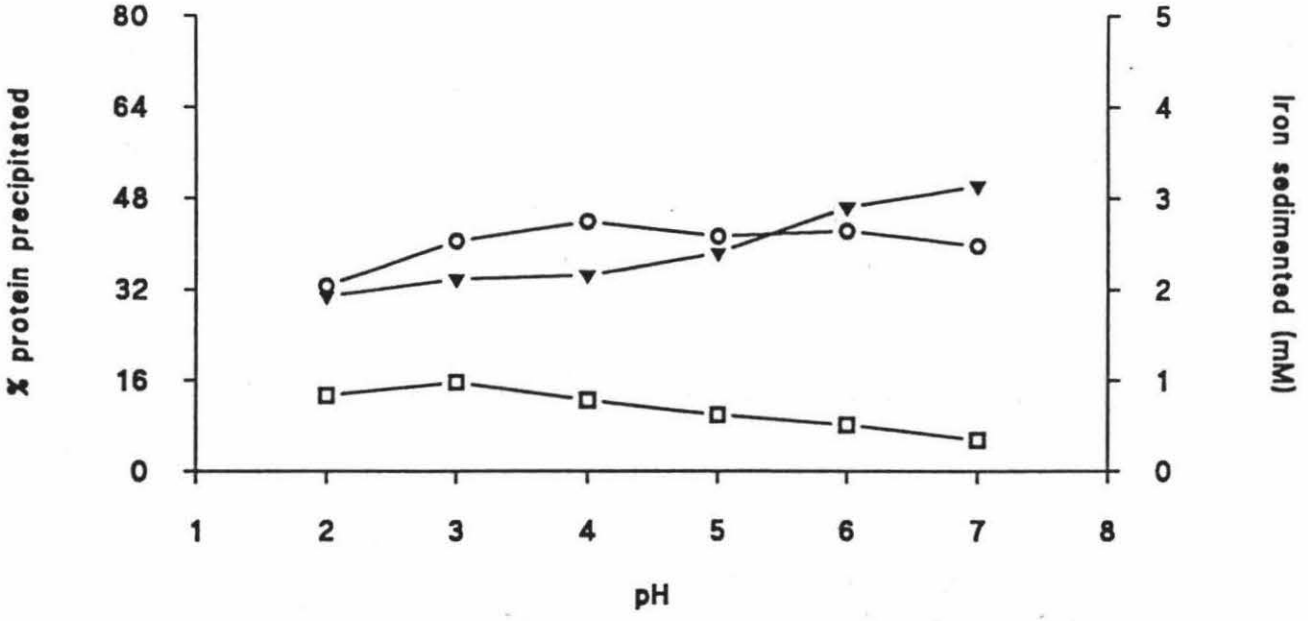


Fig 5.6 (d) Effect of pH on the solubility of albumen and iron at protein : iron ratio of 1 : 0.18. (o) protein with iron, (□) protein without iron and (▾) iron

solubility appeared to parallel changes in the pH of the system either toward or away from the isoelectric point of the individual proteins.

Solubility of WPI, without added iron, changed slightly with pH and showed a slight minimum at pH 4.0. Addition of iron decreased solubility of WPI at all pH values. In the presence of iron, solubility of WPI decreased gradually between pH 2 and 5, but increased at higher pH values. Upon addition of iron, minimum solubility was observed at pH 5 and maximum solubility at pH 2 (Fig. 5.6a).

Solubility curves for casein (Fig. 5.6b) indicate that the solubility of casein with and without added iron followed similar pattern with minimum solubility at pH 4. There was a large decrease in the solubility upon addition of iron. As expected, increase in pH at either side of the isoelectric pH (4.6) led to an increase in the solubility with and without added iron, but the decrease in the solubility observed at pH 7 cannot be fully explained.

Solubility of SPI varied with changes in pH and the addition of iron decreased the solubility of SPI at all pH values. Solubility curves with and without added iron were similar (Fig. 5.6c). Maximum solubility was observed at pH 6, and a minimum solubility at pH 4.

In contrast to other proteins, the solubility of albumen, with and without added iron was not significantly affected by changes in pH. Addition of iron decreased the solubility of the protein (Fig. 5.6d) at all pH values.

The changes in the solubility of proteins with pH and decreased solubility upon addition of iron observed in the present study agrees well with the findings of Rao and Rao (1975) and Nelson and Potter (1979). At the isoelectric point, the net charge on the protein molecule is zero (Walstra, 1983); consequently, the uncharged protein molecules tend to come together and form aggregates. The formation of aggregates leads to decreased

solubility (Wong, 1988).

Regardless of the protein source, there was a decrease in the solubility of iron with increase in pH (Fig. 5.6a-d). With increase in pH from 2 to 5 there was a gradual decrease in the solubility of iron, but increase in pH above 5 caused a sharp decrease in the solubility of iron. However, in the case of albumen the sharp decrease in the solubility above pH 5.0 was not observed. The decrease in the solubility at pH above 5.0 may be due to increased association of iron with protein and\or due to formation of insoluble hydroxides. At pH 7.0, iron forms insoluble iron hydroxide (Leigh and Miller, 1983).

5.6. Effect of pH on the binding of iron to protein

Figure 5.7 (a,b,c,d) illustrates the effect of pH in the range 2.0 - 7.0 on the binding of iron to the insoluble and soluble fractions of the four proteins. The binding curves varied considerably with the protein source.

The binding of iron to the insoluble fraction of WPI was not affected significantly with increase in pH between 2 and 4 but further increase in pH from 4.0 to 7.0 led to a gradual increase in the binding (Fig. 5.7a). The amounts of iron bound to the soluble fraction remained essentially unaffected as the pH was increased from 2 to 5; but between pH 5 and 7, there was a gradual decrease in binding. Thus, the distribution of iron between the soluble and insoluble fractions was affected by pH; at pHs below 5.0, more iron was bound by the soluble fraction whereas above pH 5.0, the insoluble fraction bound more iron (Fig. 5.7a).

The binding of iron to the insoluble and soluble fractions of casein as a function of pH is shown in Fig. 5.7 (b). The amounts of iron bound by the insoluble fraction decreased slightly from ~34 mg to ~25 mg between pH 2 and 4 but increased markedly between pH 5 and 6. At pH 7.0, there was a decrease in the amounts of iron bound to the insoluble fraction. Maximum binding was observed at pH 6.0 (126 mg/g protein). Studies by Nelson and

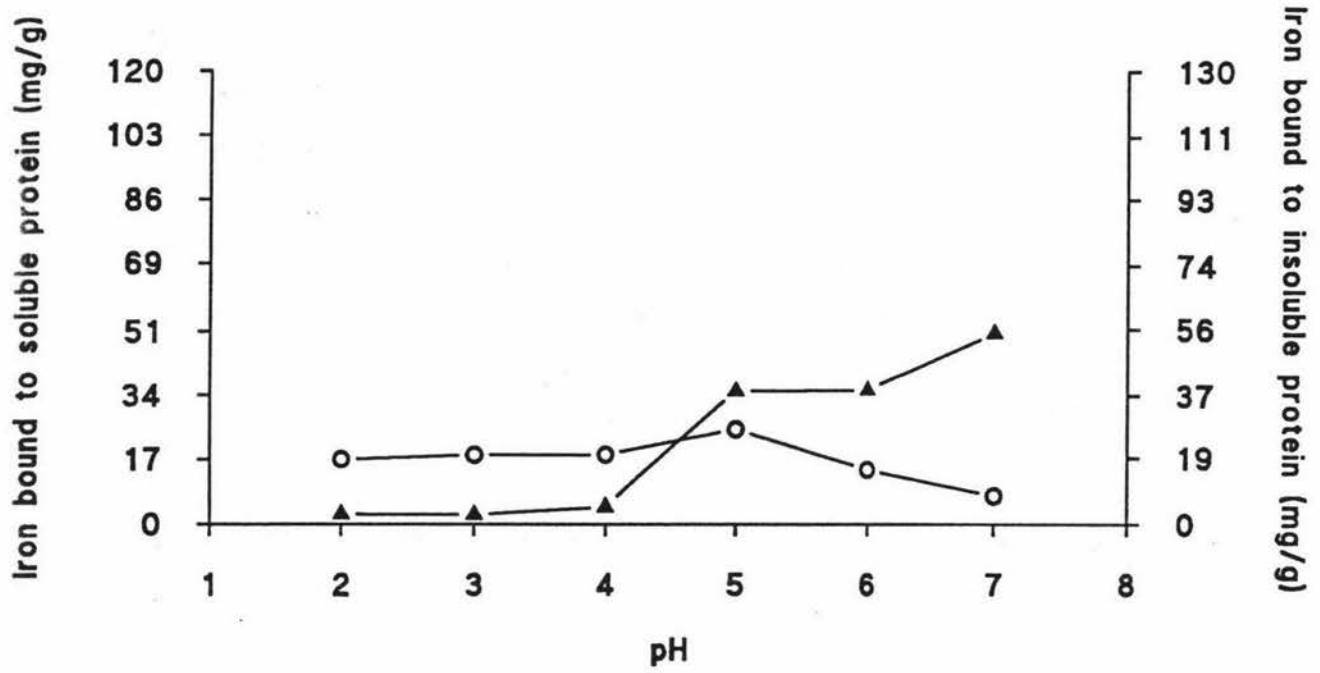


Fig 5.7 (a) Effect of pH on the binding of iron to the soluble (o) and insoluble (▲) fractions of WPI at 100 mg iron/g protein.

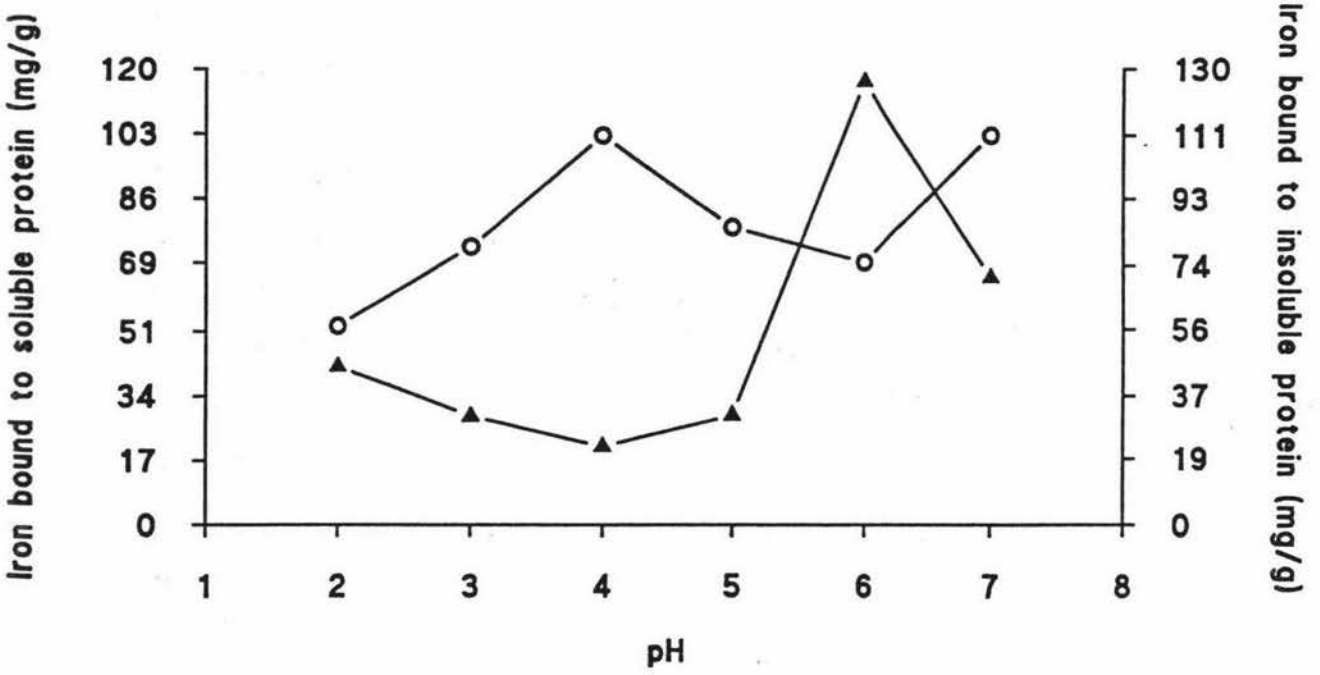


Fig 5.7 (b) Effect of pH on the binding of iron to the soluble (o) and insoluble (▲) fractions of casein at 160 mg iron/g protein.

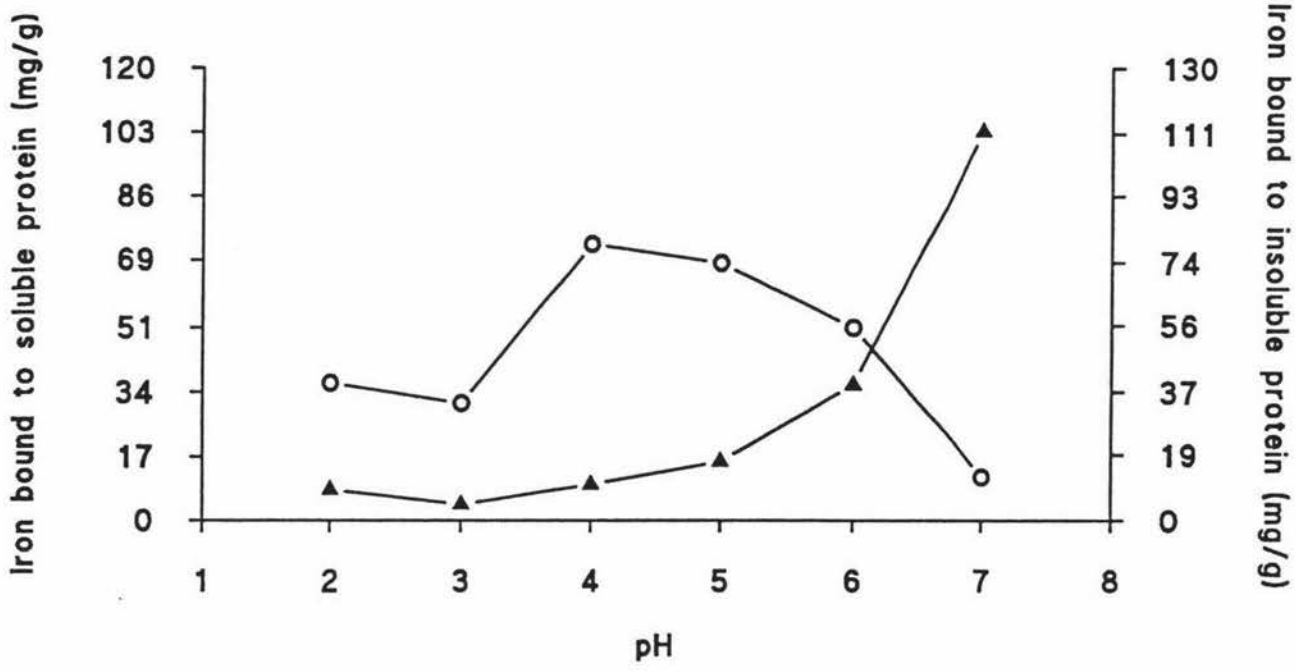


Fig 5.7 (c) Effect of pH on the binding of iron to the soluble (o) and insoluble (▲) fractions of SPI at 160 mg iron/g protein.

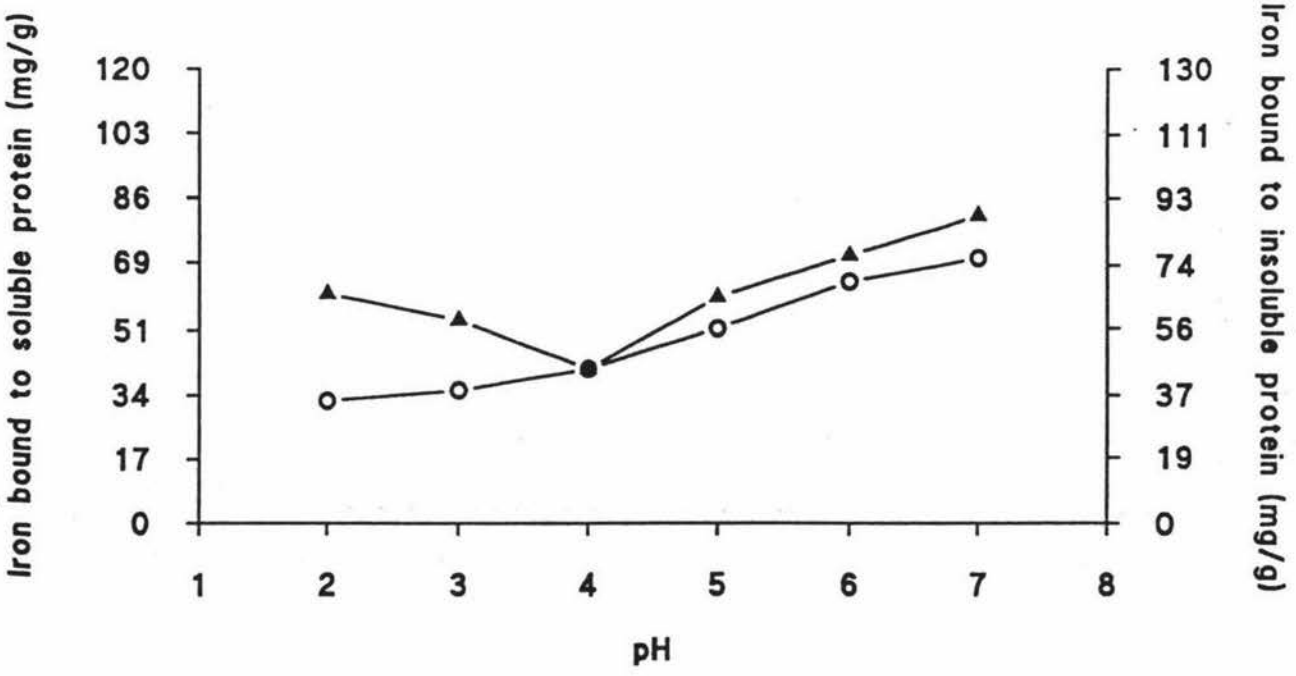


Fig 5.7 (d) Effect of pH on the binding of iron to soluble (o) and insoluble (▲) fractions of albumen at 160 mg iron/g protein.

Potter (1979) showed that casein bound maximum iron at pH 5 and 6, which partly agrees with the findings of the present study. The soluble fraction of casein bound increasing amounts of iron when the pH was increased between 2 and 4. There was a decline in binding between pH 5 and 6 and a sharp increase at pH 7 (Fig. 5.7b).

The insoluble fraction of SPI bound increasing amounts of iron with increase in pH, except at pHs 2 and 3 (Fig. 5.7c). The increase in pH from 2 to 3 caused a decrease in the binding, *i.e.* 8.9 mg iron/g protein to 4.7 mg iron/g protein. The binding increased gradually between pH 3 and 6 but at pH 7, there was a much greater increase in the binding. Maximum binding by the insoluble fraction was observed at pH 7 (111 mg iron\g protein) whereas least amount of iron (4.7 mg iron/g protein) was bound when the pH of the system was 3. Similar results have been reported by Nelson and Potter (1979a). Binding by the soluble fraction of SPI showed an irregular binding curve as the pH was raised sequentially between 2 and 7 (Fig. 5.7c). There was a slight decrease in the binding when the pH was raised from 2 to 3 followed by a sharp increase at pH 4. Maximum binding was observed at pH 4 (73 mg iron/g protein) and further increase in pH up to 7 caused a steady decrease in the binding, with least binding at pH 7 (11.6 mg iron\g protein).

Binding of iron by the insoluble fraction of albumen decreased slightly with increase in pH between 2 and 4. Increasing the pH from 4 to 7 caused a steady increase in binding (Fig. 5.7d). Maximum iron (88 mg) was bound at pH 7 and minimum iron (44 mg) at pH 4. The binding of iron to the soluble fraction of albumen (Fig. 5.7d) continuously increased with increase in pH with 32 mg iron bound/g protein at pH 2 and 70 mg iron bound/g protein at pH 7.

The sharp increases and decreases in the binding of iron to proteins with change in pH agrees with the finding of several researchers (Nelson and Potter, 1979; Rizk and Clydesdale, 1983; Patocka and Jelen, 1991).

The present results show that there was a critical pH region in which a small change in pH results in a large change in the amounts of bound iron. The critical pH range varied with the protein and also on whether the binding was to the soluble or insoluble fraction of protein.

It is generally believed that the changes in pH affect the complex formation between metal ions and proteins as hydrogen ions compete with the metal ion for protein (Wilcox, 1962). At low pHs, the reactive side chains of amino groups tend to become protonated which decreases their affinity for cations, thus reducing their complexation with the protein. When the pH is increased, the same side chains would have a negative charge and tend to complex with cations. In addition, the change in the pH of the system can lead to reversible conformational changes in proteins thereby altering their metal binding capacity (Patocka and Jelen, 1991). Hiraoka *et al.* (1980) reported that proteins have two binding sites for cations at pHs above the isoelectric point, some ions are tightly bound (forming ion pairs) and other ions act as counter ions shielding the charge. The effects of pH on binding of metal ions to casein (Dickson and Perkins, 1971; Nelson and Potter, 1979), soya proteins (Rizk and Clydesdale, 1980; Kroll, 1984; Norsworthy and Caldwell, 1987) and whey proteins (Patocka and Jelen, 1991) have been reported in the literature.

EFFECT OF ADDITION OF CHELATING AGENTS ON THE BINDING OF IRON TO WHEY PROTEIN, CASEIN, SOYA PROTEIN AND EGG ALBUMEN

Iron-binding isotherms for whey protein isolate (WPI), casein, soya protein isolate (SPI) and albumen indicated that maximum binding was approached at 100 mg added iron/g WPI, 160 mg iron/g casein and SPI, and 180 mg iron/g albumen. Therefore, the effects of chelating agents (ascorbic acid and citric acid) on binding of iron to the above proteins were investigated at these protein-iron ratios.

6.1. Effect of addition of chelating agents on the solubility of protein and iron

Addition of ascorbic acid or citric acid increased the solubility of all four proteins (Fig. 6.1 a,b,c,d). In general, at all concentrations of added chelating agents, the solubilities of the protein-iron mixtures were lower than the solubilities of proteins containing no iron.

Solubility of WPI (with no added iron) increased with the additions of citric acid or ascorbic acid in the range 0 - 400 mg/g protein. There was no significant differences between the effects of ascorbic acid and citric acid. A similar trend was observed in the solubility of WPI in the presence of iron, although the extent of increase was somewhat smaller (Fig. 6.1a).

As observed in the case of WPI, the solubility of casein (containing no iron) increased with the additions of ascorbic or citric acid, with no significant differences between ascorbic and citric acid (Fig. 6.1b). In the presence of iron, addition of ascorbic acid to casein caused a greater increase in the solubility of casein than the addition of citric acid, the difference being greater at higher concentrations of the chelating agents (Fig. 6.1b).

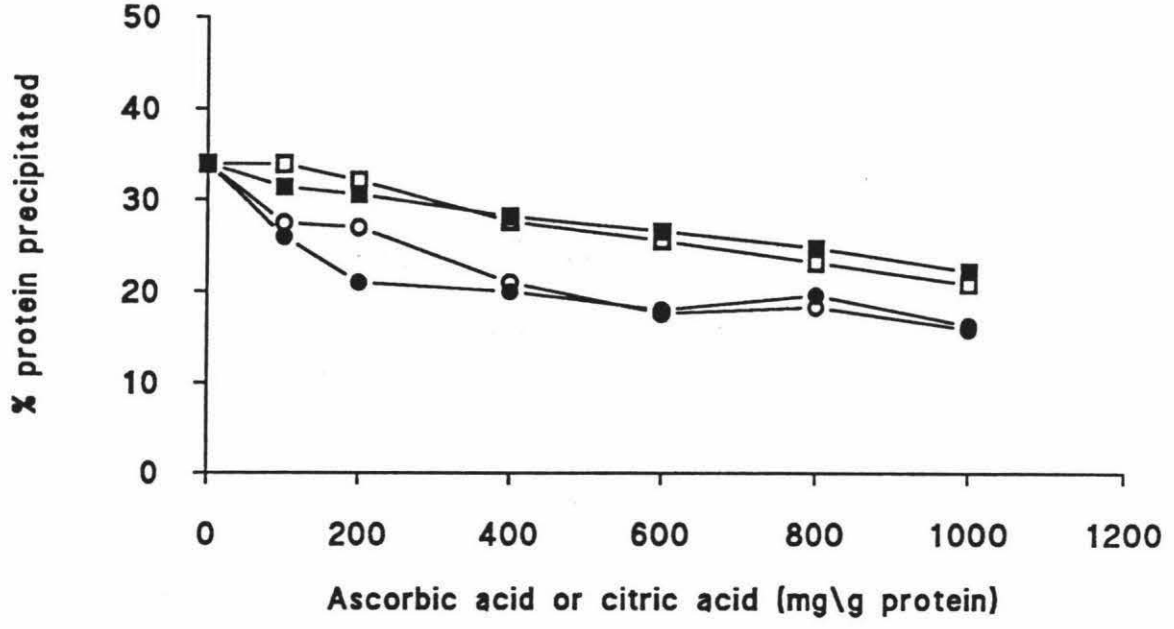


Fig 6.1 (a) Effect of addition of ascorbic acid or citric acid on the solubility of WPI at pH 6.6. WPI (control; no added iron): Ascorbic acid (●), citric acid (○). WPI–iron mixture : Ascorbic acid (■), citric acid (□)

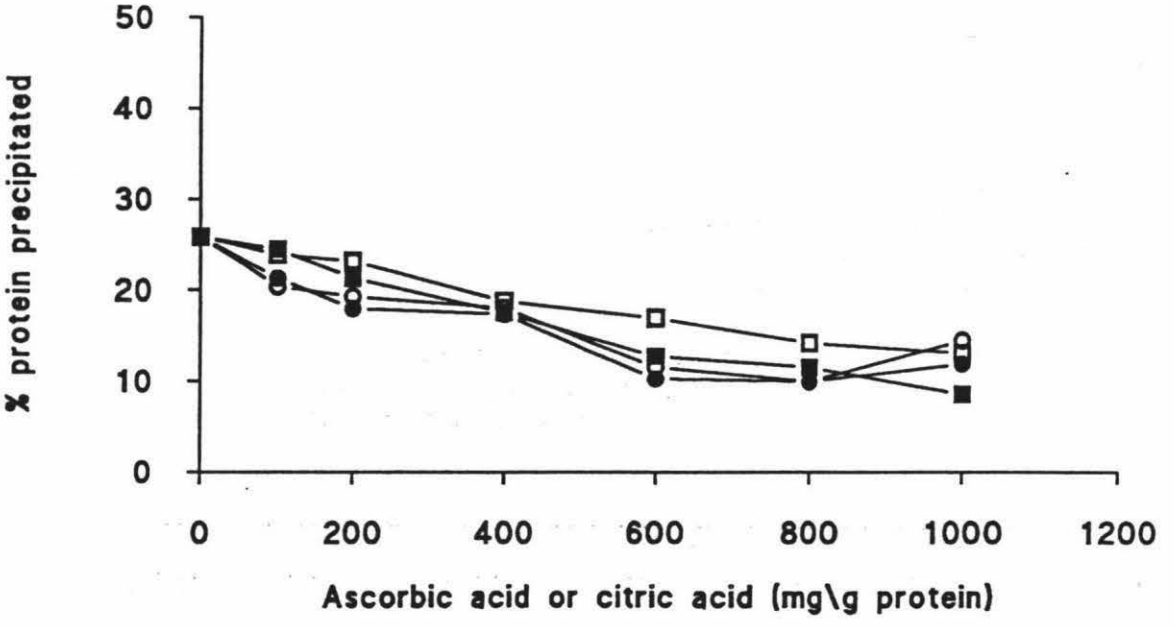


Fig 6.1 (b) Effect of addition of ascorbic acid and citric acid on the solubility of Casein at pH 6.6. Casein (control; no added iron) : Ascorbic acid (●), citric acid (○). Casein–iron mixture : Ascorbic acid (■), citric acid (□).

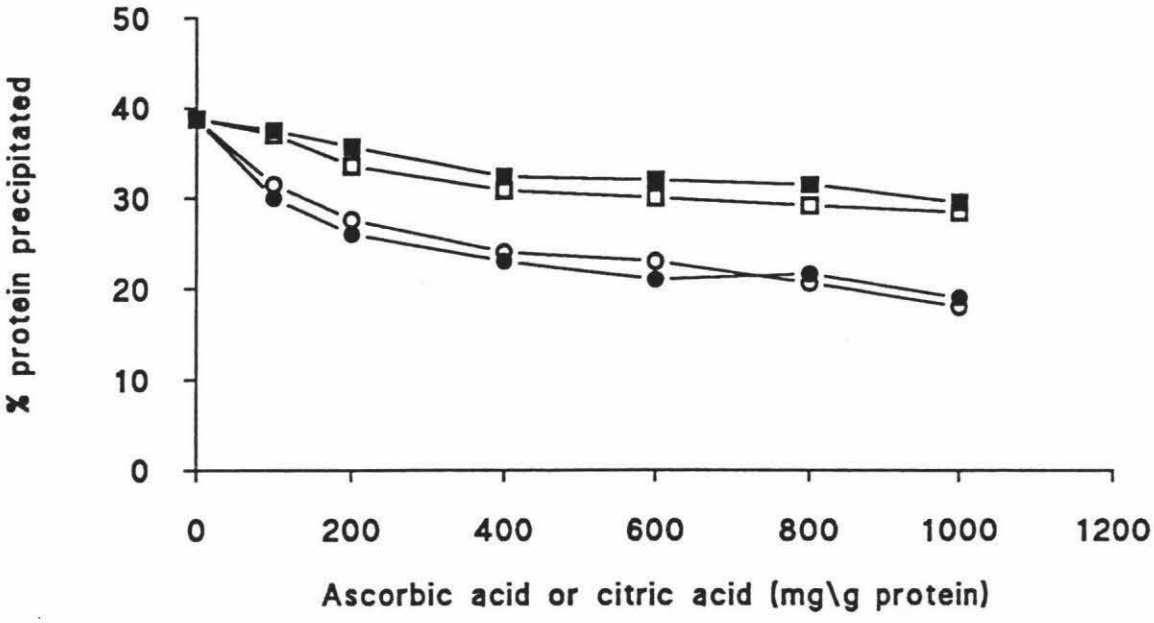


Fig 6.1 (c). Effects of addition of ascorbic acid and citric acid on the solubility of SPI at pH 6.6. SPI (control; no added iron) : Ascorbic acid (●), citric acid (○). SPI–iron mixture : Ascorbic acid (■), citric acid (□)

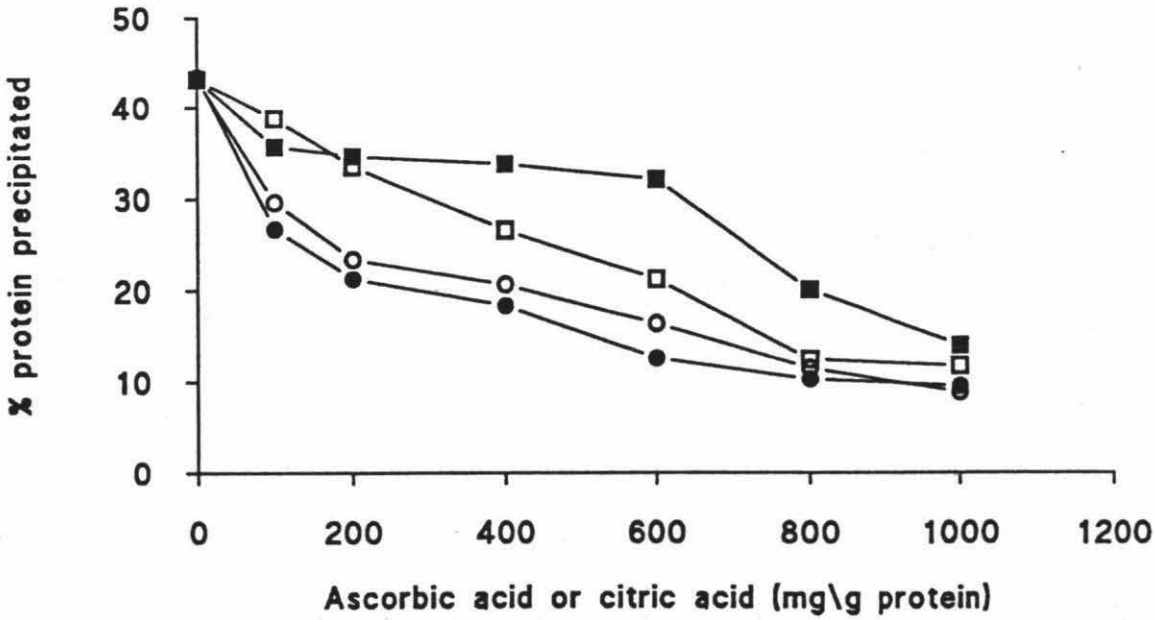


Fig 6.1 (d). Effects of addition of ascorbic acid or citric acid on the solubility of albumen at pH 6.6. Albumen (control; no added iron) : ascorbic acid (●), citric acid (○). Albumen–iron mixture : Ascorbic acid (■), citric acid (□).

The changes in the solubility of SPI with addition of ascorbic or citric acid were quite similar to those observed for WPI (Fig. 6.1c).

In the absence of iron, the solubility of albumen increased with the additions of ascorbic acid and citric acid, with no significant differences between ascorbic acid and citric acid. However, in the presence of iron, there were significant differences; at concentrations above 400 mg acid/g protein, the solubility was greater in the presence of citric acid, the difference decreasing somewhat with increase in the acid concentration. The greatest difference (11%) was observed at a concentration of 600 mg/g protein (Fig. 6.1d).

Binding of iron to the protein molecules may initiate aggregation by shielding of the charges on the protein surface, hence resulting in a decrease in the solubilities of proteins in the presence of iron. Upon addition of citric acid or ascorbic acid, the protein-iron bonds are disrupted, as these acids have higher affinity for iron when compared to proteins. This may explain increased solubility of the proteins in the presence of acids.

There was a decrease in the sedimentable iron with the additions of citric acid and ascorbic acid, regardless of the protein (Fig. 6.2 a,b,c,d). There was only a slight variation in the amount of iron sedimented when the solubilizing effects of both acids were compared. When the concentration of ascorbic acid was increased from 0 mg to 1000mg\g protein, the amounts of iron in the sediment decreased from 0.9 mM to 0.3 mM, 1.98 mM to 0.26 mM, 1.14 mM to 0.2 mM and 3.169 mM to 0.302 mM for WPI, casein, SPI and albumen, respectively. In the presence of citric acid (in similar amounts) the amounts of iron in the sediment decreased from 0.913 mM 0.214 mM, 2.11 mM to 0.198 mM, 1.14 mM to 0.125 mM and 3.16 mM to 0.17 mM for WPI, casein, SPI and albumen, respectively (Fig. 6.2 a,b,c,d).

The decrease in the amount of iron in the sediment suggests that both acids have the ability of increasing the solubility of iron. Previous researchers

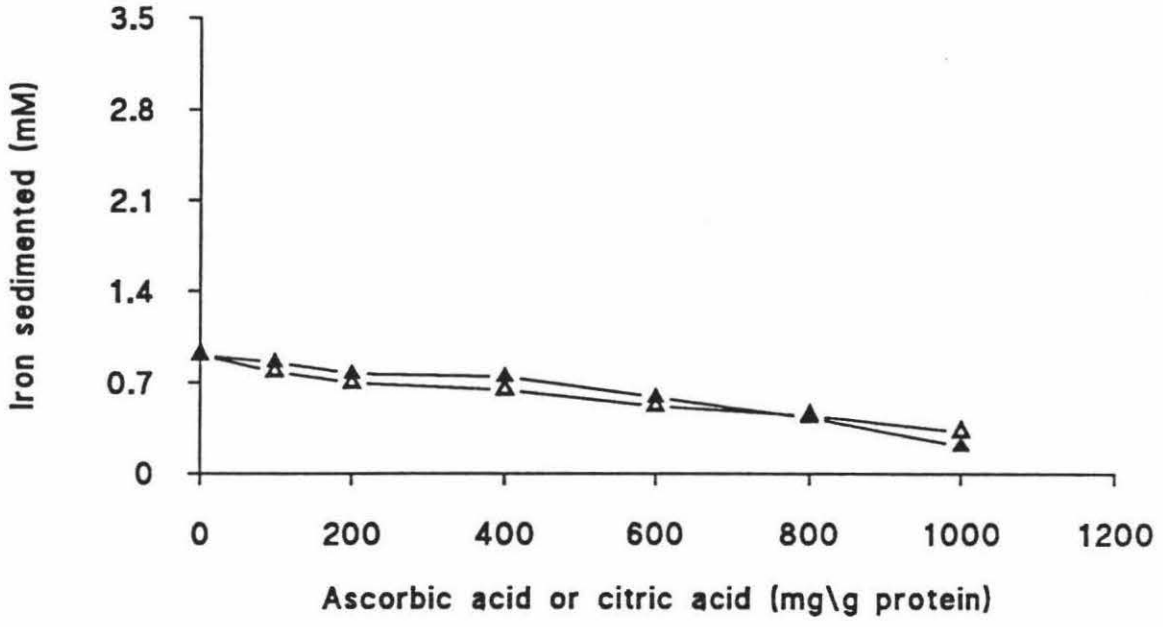


Fig 6.2 (a) Solubility of iron in WPI–iron mixture, (△) with citric acid, and (▲) with ascorbic acid.

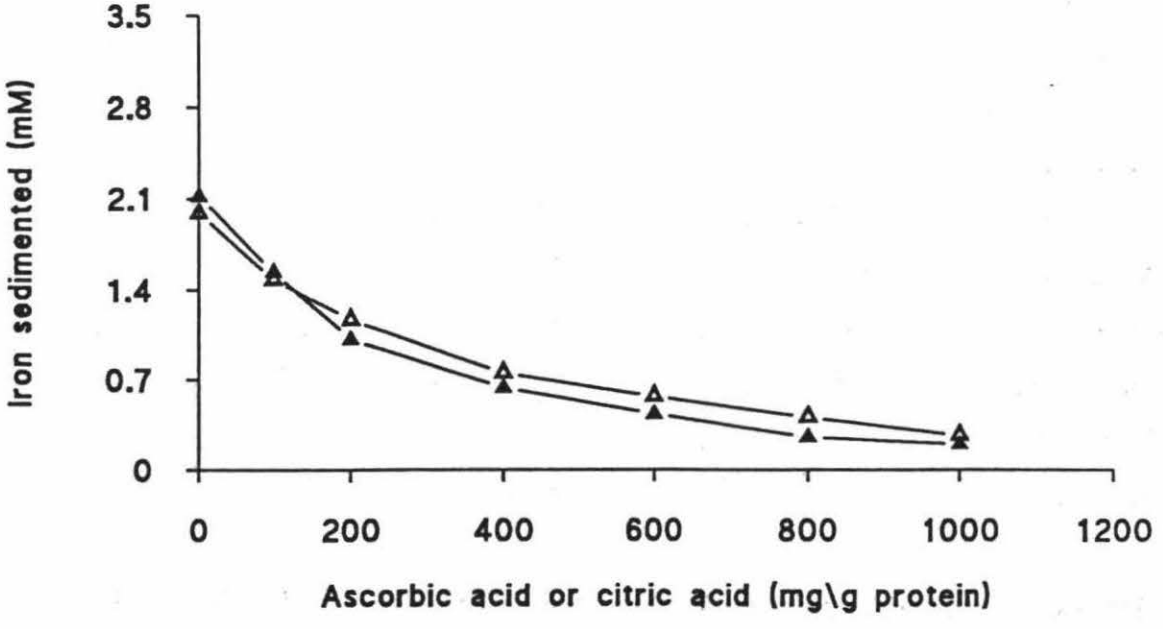


Fig 6.2 (b) Solubility of iron in Casein–iron mixture,(△) with citric acid and (▲) with ascorbic acid.

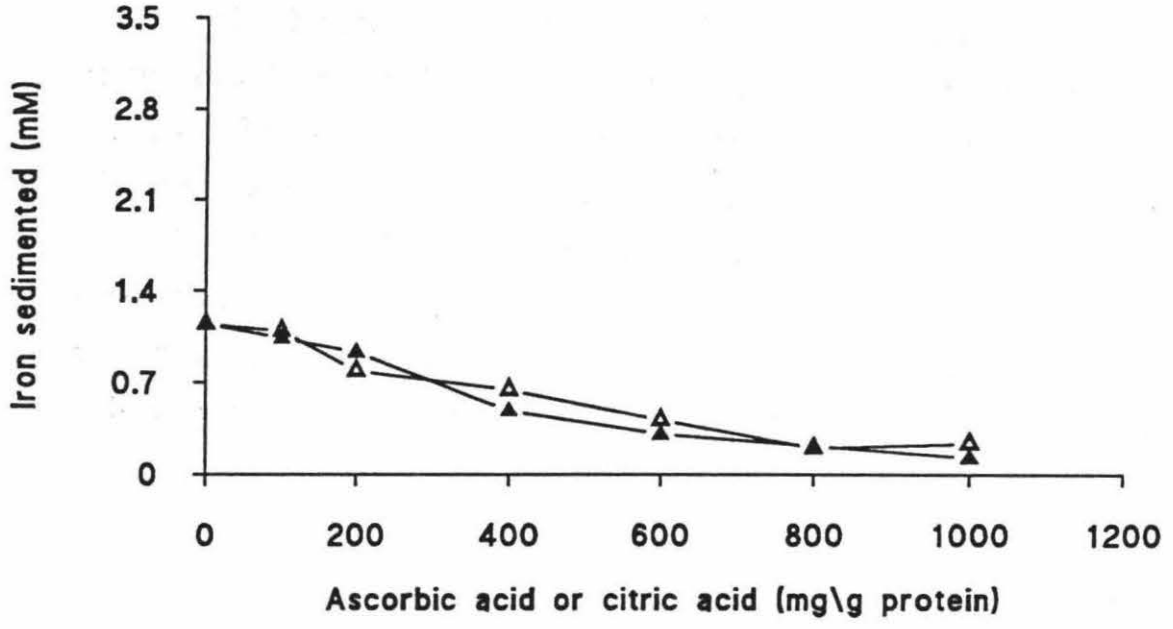


Fig 6.2 (c). Solubility of iron in SPI–iron mixture, (△) with citric acid and (▲) with ascorbic acid.

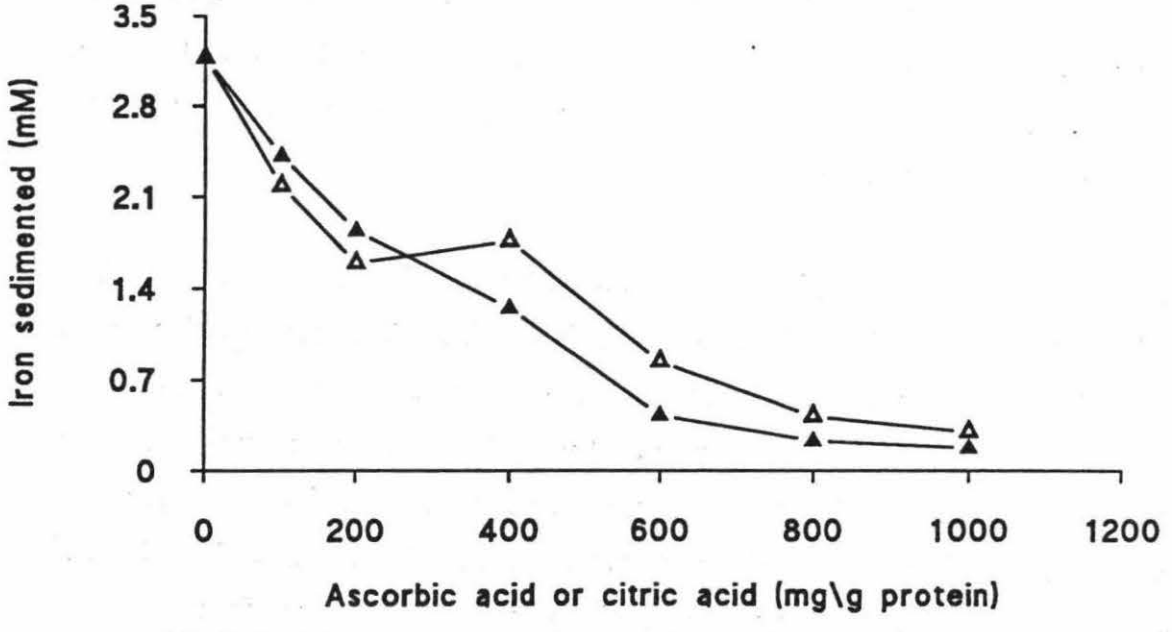


Fig 6.2 (d) Solubility of iron in albumen–iron mixture, (△) with citric acid and (▲) with ascorbic acid.

(Kojima *et al.*, 1981; Camire and Clydesdale., 1982; and Rizk and Clydesdale, 1983) found increased solubility of iron in the presence of acids. It was also found in the present study that the solubilization effect was dependent on acid concentration or iron:acid ratio, which agrees with the findings of the above mentioned researchers. The decreased iron sedimentation might be due to two reactions; (i) upon addition of acid, iron, which would bind to the insoluble fraction of the protein in the absence of acid, forms a soluble low-molecular weight complex with acid, or (ii) chelating agents might be successful in breaking the bond between iron and protein and thus releasing iron (Clydesdale, 1983).

6.2. Effect of chelating agents on the binding of iron to proteins

6.2.1 Binding to the insoluble fraction

Addition of ascorbic acid or citric acid decreased the binding of iron to the insoluble fraction of WPI (Fig. 6.3a). With increase in concentration of ascorbic acid from 0 - 1000 mg/g protein, there was a steady decrease in the amount of iron bound to the insoluble fraction *i.e.* from 30 mg to 10.7 mg/g protein. When citric acid was added in similar amounts, the binding decreased from 30 mg to 17.3 mg/g protein. The results showed that at lower concentrations (0-800 mg/g protein), both acids had similar effect on the binding of iron, but at a higher concentration (1000 mg\g protein), citric acid was slightly less effective (Fig. 6.3a).

The amount of iron bound by the insoluble fraction of casein decreased when citric acid and ascorbic acid were added (Fig. 6.3b). The binding decreased from 85 mg to 25 mg iron\g protein when ascorbic acid was added in the concentration range of 0-1000 mg\g protein. When citric acid was added in similar amounts the binding decreased from 85 mg to 22 mg\g protein. There was no significant difference between the effects of citric acid and ascorbic acid; the protein bound essentially similar amounts of iron (Fig. 6,3b).

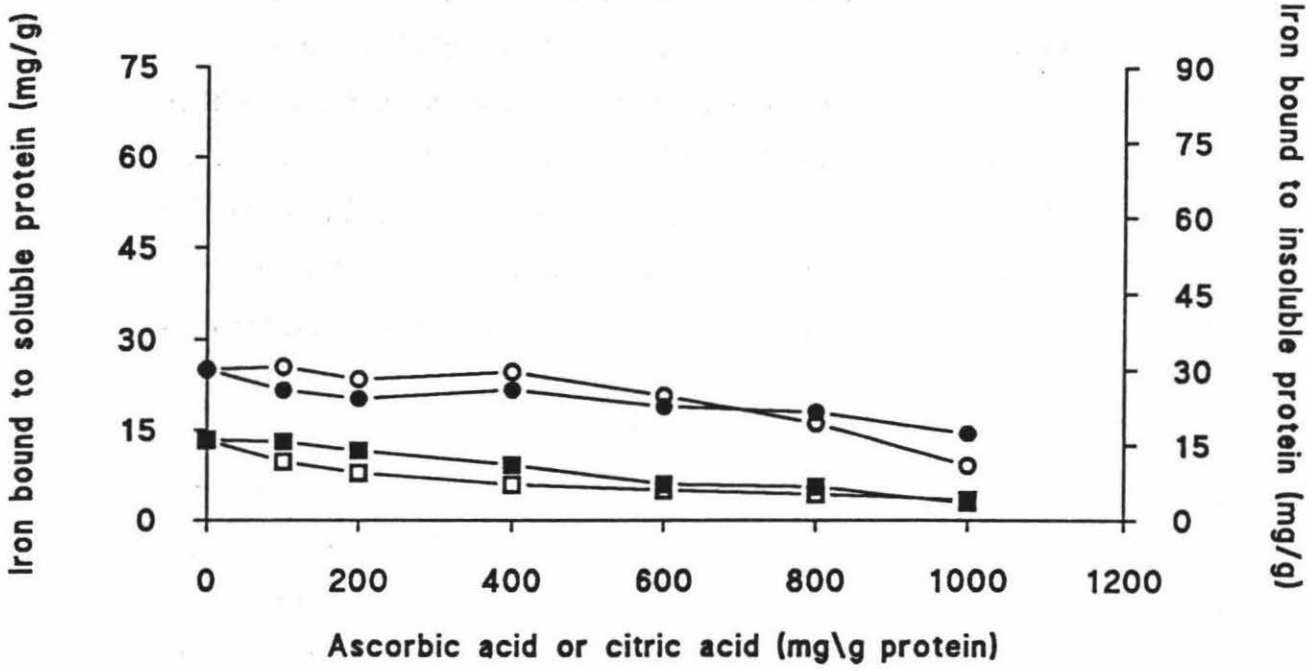


Fig 6.3 (a) Effect of addition of ascorbic acid (o, □) and citric acid (●, ■) on the binding of iron to soluble (□, ■) and insoluble (o, ●) fractions of WPI

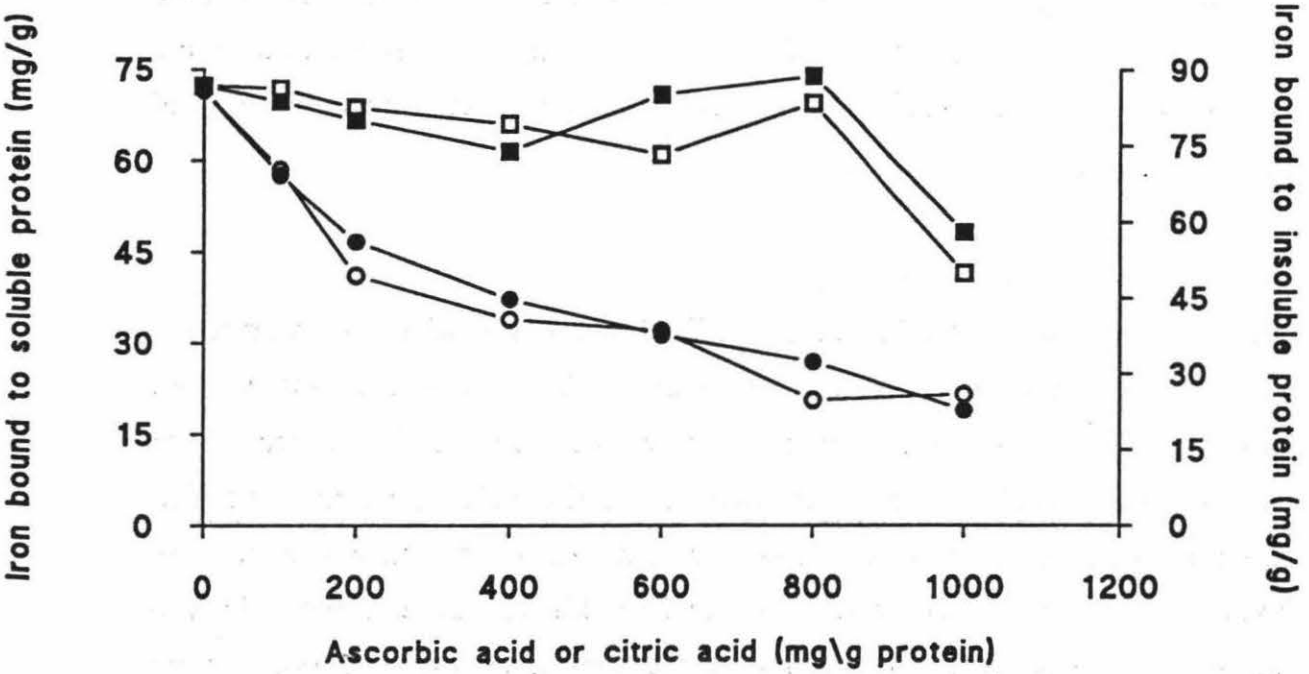


Fig 6.3 (b) Effect of addition of ascorbic acid (o, □) and citric acid (●, ■) on the binding of iron to soluble (□, ■) and insoluble (o, ●) fractions of casein

There was a significant decrease in the amount of iron bound by the insoluble fraction of SPI on addition of ascorbic acid and citric acid (Fig. 6.3c). When the concentration of ascorbic acid was sequentially increased from 0 - 1000 mg/g protein, the binding decreased from 32 mg to 4.7 mg/g protein. In the presence of citric acid (in similar amounts), iron bound/g of insoluble fraction of SPI decreased from 32 mg to 9.1 mg. At all other concentrations of acid, the amount of iron bound by protein in the presence of citric acid and ascorbic acid were not significantly different (Fig. 6,3c).

The binding by the insoluble fraction of albumen decreased in the presence of both ascorbic acid and citric acid, the extent of which increased with increase in concentration of acid (Fig. 6.3d). There was a significant difference in the amount of iron bound by albumen when comparison was made between the two chelating agents. When both acids were added in the concentration range of 100-200 mg/g protein, albumen bound more iron in the presence of ascorbic acid when compared to citric acid. Above this concentration of acid, albumen bound significantly less iron in the presence of ascorbic acid than in the presence of citric acid. At 600 mg added citric acid or ascorbic acid, the amount of iron bound in the presence of ascorbic acid was 12 mg whereas in the presence of citric acid it was 38 mg/g of insoluble albumen (Fig. 6,3d).

6.2.2. Binding to the soluble fraction

Binding of iron to the soluble fraction of WPI decreased with the additions of ascorbic acid and citric acid (Fig. 6.3a). With the increase in the concentration of added acid, there was a gradual decrease in the amount of bound iron. When 0-1000 mg citric acid or ascorbic acid/g protein was added binding decreased from 13 mg to ~3 mg. As can be seen in Figure 6.3 (a), both citric acid and ascorbic acid had similar effect on the binding of iron to the soluble fraction of WPI, except at the concentration of 100-400 mg/g protein. At these concentrations, WPI bound slightly more iron in the presence of citric acid than in the presence of ascorbic acid.

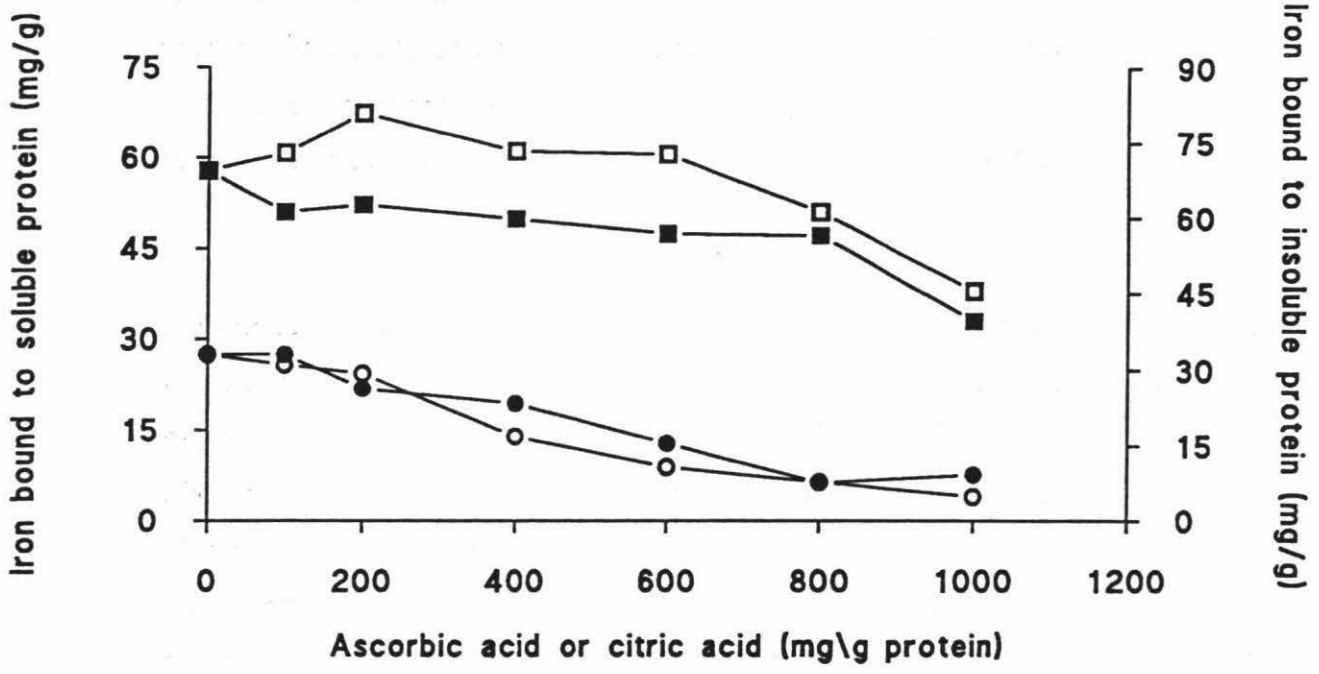


Fig 6.3 (c) Effect of addition of ascorbic acid (o, □) and citric acid (●, ■) on the binding of iron to soluble (□, ■) and insoluble (o, ●) fractions of SPI

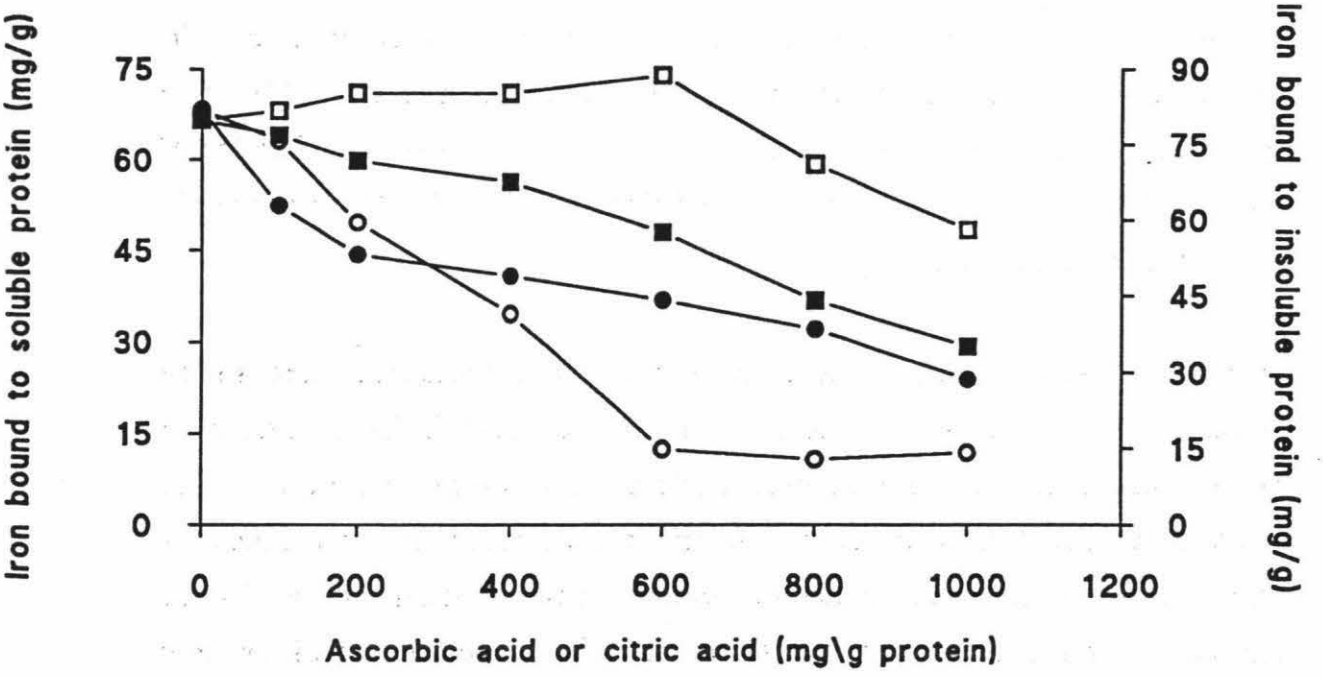


Fig 6.3. (d) Effect of addition of ascorbic acid (o, □) and citric acid (●, ■) on the binding of iron to soluble (□, ■) and insoluble (o, ●) fractions of albumen

The binding of iron to the soluble fraction of casein did not follow any consistent pattern (Fig. 6.3b) with increase in the concentration of ascorbic acid and citric acid from 0 to 800 mg/g protein. However, there was a marked decrease in binding when the concentration of the acids was increased from 800 to 1000 mg/g protein.

Addition of citric acid decreased the binding of iron by the soluble fraction of SPI. There was a gradual decrease in binding when the concentration of citric acid was increased. The binding decreased from 57 mg to 32 mg when the concentration of citric acid was increased sequentially from 0 to 1000 mg/g protein. The effect of addition of ascorbic acid on the binding of iron to the soluble fraction of SPI was dependent on the concentration of acid. The binding increased slightly at a concentration of up to 200 mg/g protein. In contrast, when the concentration of ascorbic acid was increased from 400 to 1000 mg/g protein, the amount of iron bound/g of soluble fraction of SPI decreased from 60 mg to 37 mg (Fig. 6.3c).

Addition of citric acid caused a decrease in binding by the soluble fraction of albumen, the effect increasing with increase in the concentration of acid. In contrast, the addition of ascorbic acid in the concentration range of 100 - 600 mg/g protein led to a slight increase in the binding by the soluble fraction of albumen; the binding decreased upon addition of 800 and 1000 mg ascorbic acid/g protein (Fig. 6.3d).

Overall, the results show that both citric acid and ascorbic acid increase the solubility of iron and have the ability to remove and solubilize iron from an insoluble complex, which agrees with the findings of Kojima *et al* (1981). The results also show that solubilization of iron from complexes, by the acids is concentration dependent and also dependent on the protein source. Under most experimental conditions, there seemed to be no significant differences between the effects of added citric acid and ascorbic acid. Similar results were reported by earlier researchers (Kojima *et al.*, 1981; Camire and

Clydesdale, 1982; Rizk and Clydesdale, 1983) using different protein preparations. Kojima *et al.* (1981) found that increasing ascorbic acid concentration in a bean (pinto) suspension from 0 to 10 mM increased soluble iron concentration from 10 to 50%.

The results indicate that the solubilization effect of both acids depends on the protein to acid ratio and also on the ratio of iron to acid, which is essentially in agreement with the results reported by previous investigators (Lynch and Cook, 1980; Kojima *et al.*, 1981; and Bothwell *et al.*, 1982). At certain protein/acid ratios (600 to 800 mg citric acid/g casein) binding of iron by the protein was higher when compared to the binding in the absence of acid. Earlier Warner and Weber (1953) and Spiro (1967) found that citrate forms polymerized complex with iron at pH 6.0 and 7.0 and this polymer formation is not favoured in the presence of excess citrate. This could explain the decline in the amount of low-molecular weight iron at lower concentrations (600 to 800 mg/g protein) of citrate, and increase in the concentration of low-molecular weight iron upon increasing the concentration of citrate (1000 mg/g protein) observed in the present study.

6.3. Effect of chelating agents on the solubility of proteins at different pH values

Experiments were carried out to investigate the effect of chelating agents at a concentration of 100 mg/g protein at different pH values. In general the addition of ascorbic acid and citric acid to protein-iron mixtures caused an increase in the solubility of proteins, with no significant differences between the two acids (Fig. 6.4 a,b,c,d).

Addition of citric acid and ascorbic acid to WPI-iron mixture had no effect on its solubility at pHs between 2 and 4, but at higher pHs, a considerable increase in the solubility occurred (Fig. 6.4a).

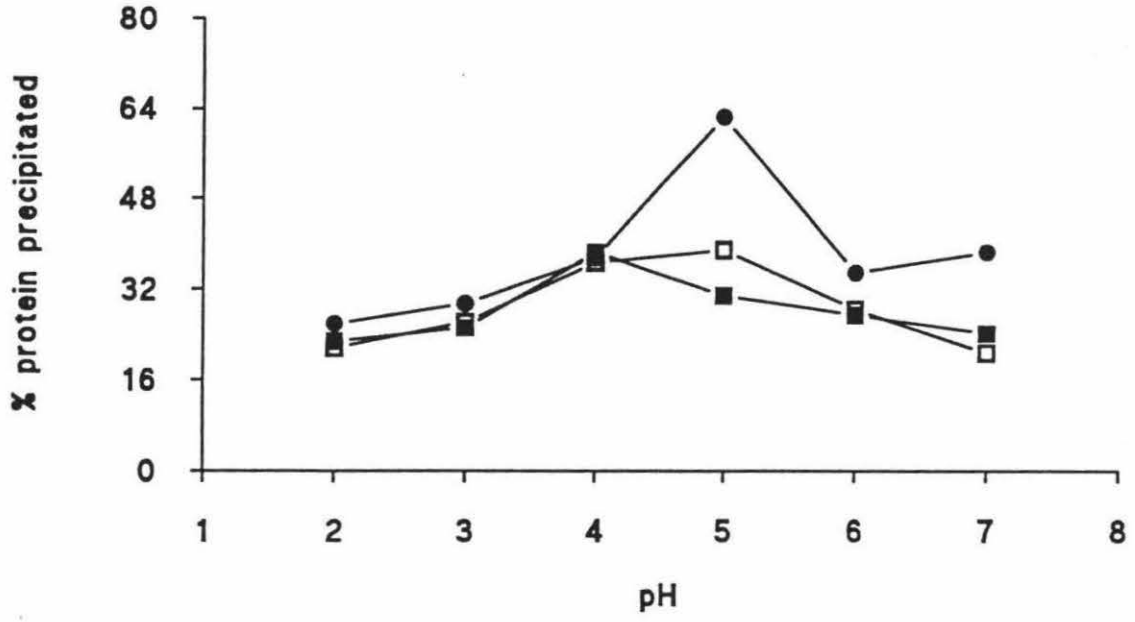


Fig 6.4 (a). Solubility of WPI in protein–iron (●), protein–iron–citric acid (□), and protein–iron–ascorbic acid (■) mixtures at different pH values

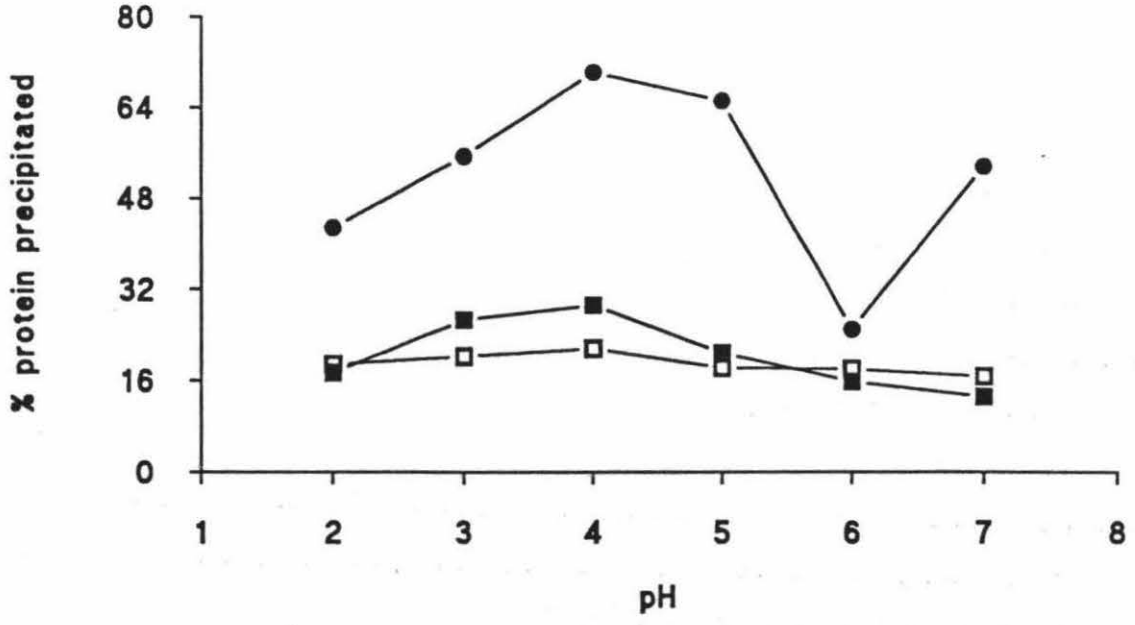


Fig 6.4 (b). Solubility of casein in casein–iron (●), casein–iron–citric acid (□), casein–iron–ascorbic acid (■) mixtures at different pH values

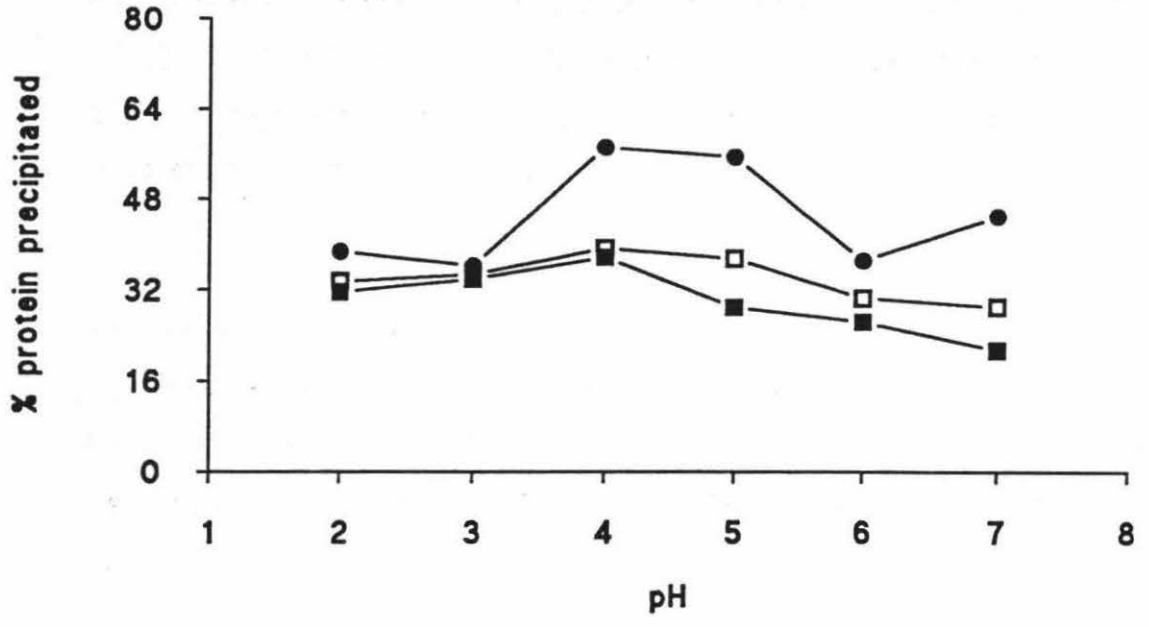


Fig 6.4 (c). Solubility of SPI in SPI–iron (●), SPI–iron–citric acid (□) SPI–iron–ascorbic acid (■) mixtures at different pH values

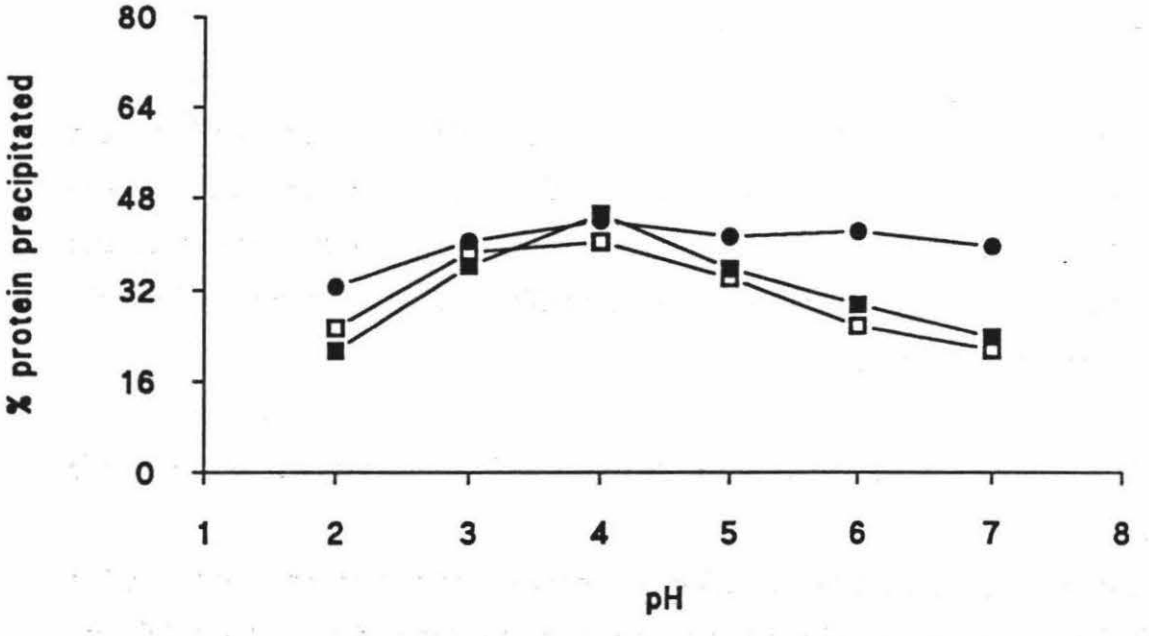


Fig 6.4 (d). Solubility of albumen in albumen–iron (●), albumen–iron–citric acid (□), albumen–iron–ascorbic acid (■) mixtures at different pH values

Upon addition of citric acid or ascorbic acid, the solubility of casein in casein-iron mixture increased at all pH values. As shown in Fig. 6.4 (b) in the presence of acids, the solubility was not significantly affected by pH. There was no significant differences between the solubilizing effects of ascorbic acid and citric acid.

In the presence of iron, addition of citric acid or ascorbic acid increased the solubility of SPI at all pH values except at pHs 2 and 3 (Fig.6.4c). The solubility curves with added acid were not significantly affected by the pH changes. As in the case of WPI and casein, there were no significant differences between the solubilizing effects of citric acid and ascorbic acid.

Addition of citric acid and ascorbic acid to albumen-iron mixture increased the solubility of albumen, especially at pH values above 5.0. Solubility curves for albumen with and without acid followed similar pattern (Fig. 6.4d). As observed in the case of other proteins, there was no significant differences between ascorbic acid and citric acid.

The results show that the effects of ascorbic acid and citric acid on the solubility of proteins is dependent on the pH and the type of protein. In the absence of acid lowest solubility was observed at isoelectric point of the individual proteins and this could be markedly improved with ascorbic acid and citric acid additions.

6.4. Effect of chelating agents on the solubility of iron at different pH values

Figures 6.5 (a,b,c,d) show the effect of addition of chelating agents on the solubility of iron in protein-iron mixture as a function of pH. The solubility of iron upon addition of ascorbic acid and citric acid varied with pH and protein source. In most cases, there was a decrease in the solubility with increase in pH from pH 2.0 to pH 7.0. The lowest solubility was at pH 7.

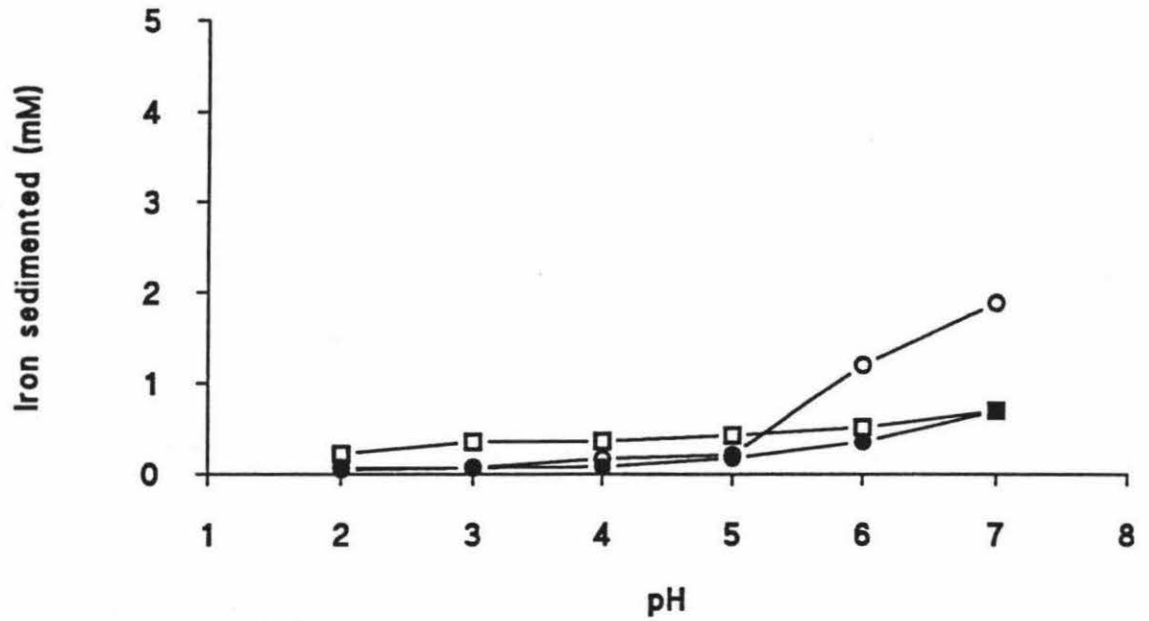


Fig 6.5 (a) Effect of pH (o), citric acid (●) and ascorbic acid (□) on the solubility of iron in WPI–iron mixture (100 mg iron and 100 mg acid/g protein)

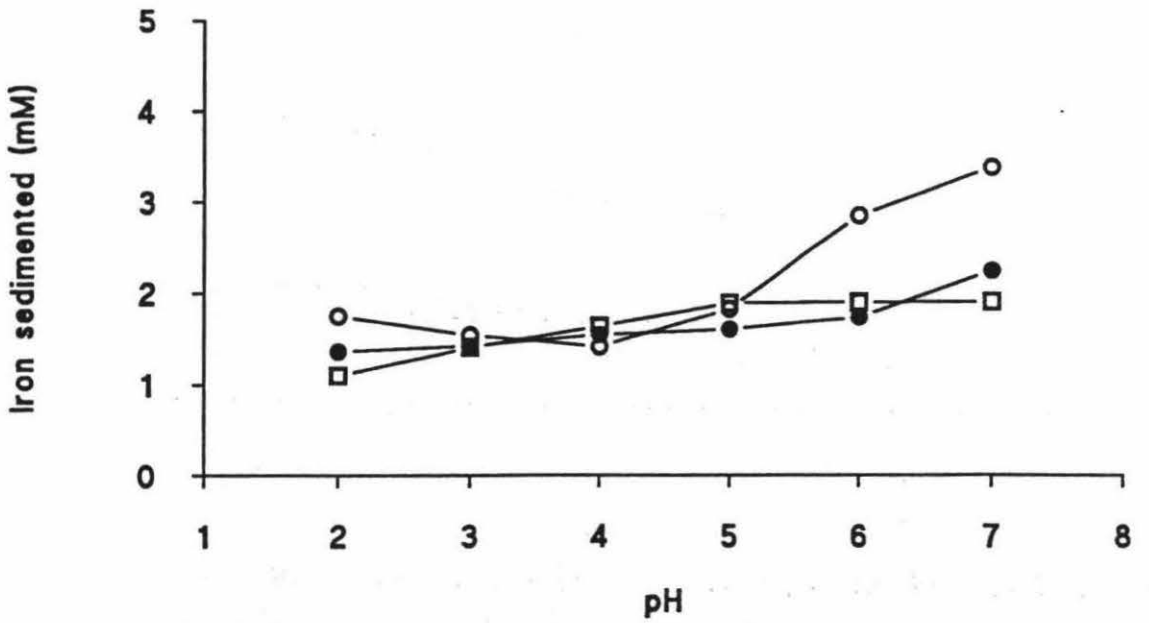


Fig 6.5 (b) Effect of pH (o), citric acid (●) and ascorbic acid (□) on the solubility of iron in casein–iron mixture (160 mg iron and 100 mg acid/g protein)

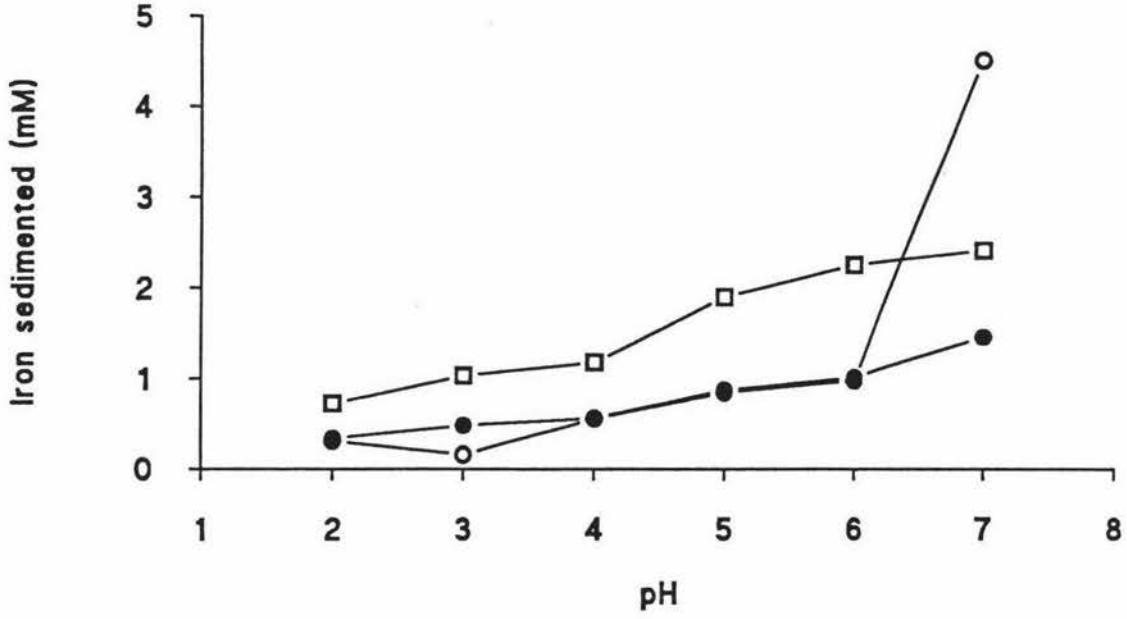


Fig 6.5 (c) Effect of pH (o), citric acid (•) and ascorbic acid (□) on the solubility of iron in SPI–iron mixture (160 mg iron and 100 mg acid/g protein)

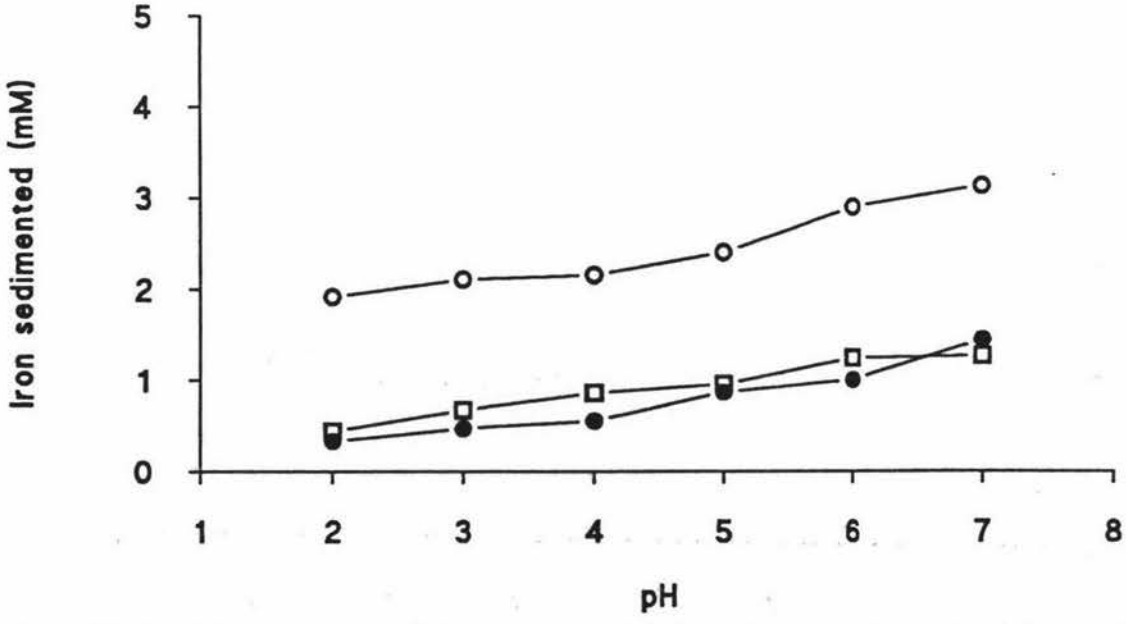


Fig 6.5 (d) Effect of pH (o), citric acid (•) and ascorbic acid (□) on the solubility of iron in albumen–iron mixture (180 mg iron and 100 mg acid/g protein)

Solubility of iron in WPI-iron mixture upon addition of citric acid and ascorbic acid changed with pH. As shown in Fig 6.5 (a) citric acid and ascorbic acid had no significant effect on the solubility of iron between pHs 2 - 5, above pH 5.0 there was an increase in the solubility of iron; there was no significant difference between ascorbic acid and citric acid.

As in the case of iron associated with WPI, upon addition of citric and ascorbic acid the solubility of iron associated with casein was pH dependent (Fig. 6.5b). There was no significant difference in the solubility of iron (with and without acid) between pHs 2 - 5, above pH 5.0 there was increase in the solubility of iron in the presence of citric acid and ascorbic acid. The differences in the solubilizing capabilities were not significant.

As observed in the case of WPI-iron mixture and casein-iron mixture the solubility of iron in SPI-iron mixture on addition of citric and ascorbic acid was dependent on the pH (Fig 6.5c). There was decrease in solubility with increase in pH. At all pH values citric acid had a greater solubilizing effect than ascorbic acid. There was a marked increase in the solubility of iron in the presence of acids at pH 7.0.

Unlike other proteins, there was an increase in the solubility of iron, with the addition of citric and ascorbic acid to albumen-iron mixture at all pH values (Fig 6.5d). There was no significant difference between the effects of citric acid and ascorbic acid. The solubility of iron did not vary significantly with variation in pH.

The significant decrease in the amount of iron sedimentable at pH 7.0 in the presence of acid when compared to the amount of iron sedimented without acid suggests that these two agents are capable of preventing the formation of insoluble hydroxides. Citric and ascorbic acid form chelates with iron thereby preventing the precipitation at pH 7 (Clydesdale, 1983). The increased solubilizing effect of ascorbic and citric acid at pH 2 observed in

some cases in the present study agrees well with the findings of Kojima *et al* (1981) and Rizk and Clydesdale (1983).

6.5. Effect of chelating agents on the binding of iron to the proteins at different pH values

6.5.1. Binding to the insoluble fraction of the proteins

The effect of addition of citric acid and ascorbic acid on the binding of iron to the insoluble fraction of WPI varied with the pH (Fig. 6,6a). Between pH 2 and 5, addition of citric acid caused no significant change in the binding, whereas in the presence of ascorbic acid, more iron was bound by the insoluble fraction of WPI. Between pH 5 and pH 7, significantly less iron was bound by the insoluble fraction of WPI in the presence of acids (Fig. 6.6a).

Binding of iron by the insoluble fraction of casein increased with the addition of citric and ascorbic acid, except at pH 6 (Fig. 6,6b). At pH 6, without added acid, casein bound 126 mg iron/g protein which decreased to ~107 mg/g protein in the presence of citric or ascorbic acid. The binding curves were similar with added citric or ascorbic acid but differed from the binding pattern of casein in the absence of acid. In the presence of acids, there was no significant difference in the amounts of iron bound when the pH was increased from 2.0 to 4.0, above this pH there was a steady increase in the binding. In the absence of citric and ascorbic acid, there was a steady decrease in the binding between pH 2 and 5 with a dramatic increase at pH 6 and a decrease at pH 7 (Fig. 6.6b).

Addition of citric and ascorbic acid led to an increase in the amounts of bound iron by the insoluble fraction of SPI, except in the presence of citric acid at pH 7 (Fig. 6.6c). At pH 7, SPI bound 111 mg iron/g protein, which decreased to 96 mg/g protein in the presence of citric acid. Binding by the insoluble fraction of SPI followed a similar pattern with and without added acid; there was an increase in the amounts of iron bound with the increase in pH from 2.0 to 7.0. A similar amount of iron was bound by SPI in the presence of

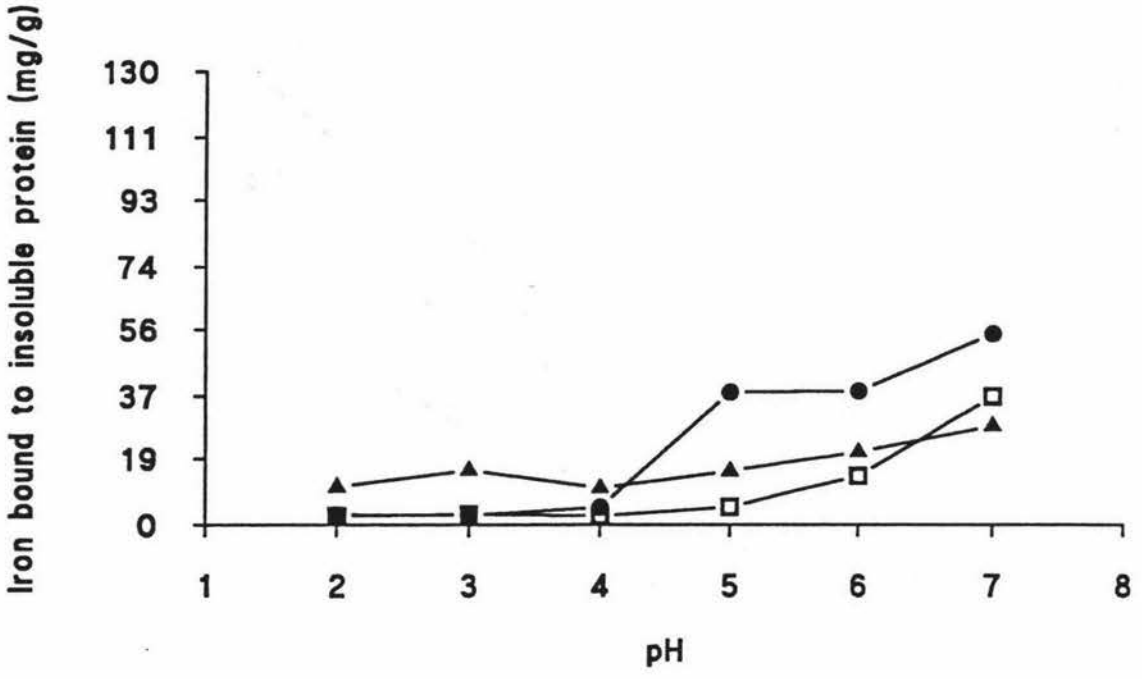


Fig 6.6 (a). Binding of iron to the insoluble fraction of WPI in the absence of acids (●) or in the presence of citric acid (□) and ascorbic acid (▲) at different pH values

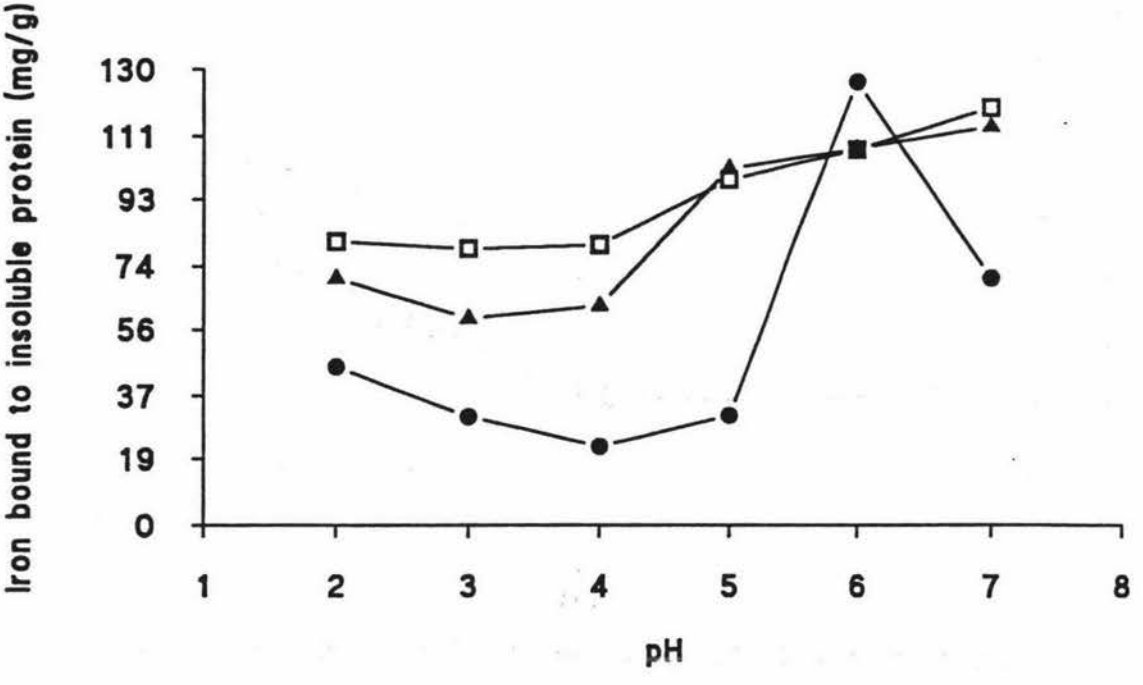


Fig 6.6 (b). Binding of iron by the insoluble fraction of casein in the absence of acids (●) or in the presence of citric acid (□) and ascorbic acid (▲) at different pH values

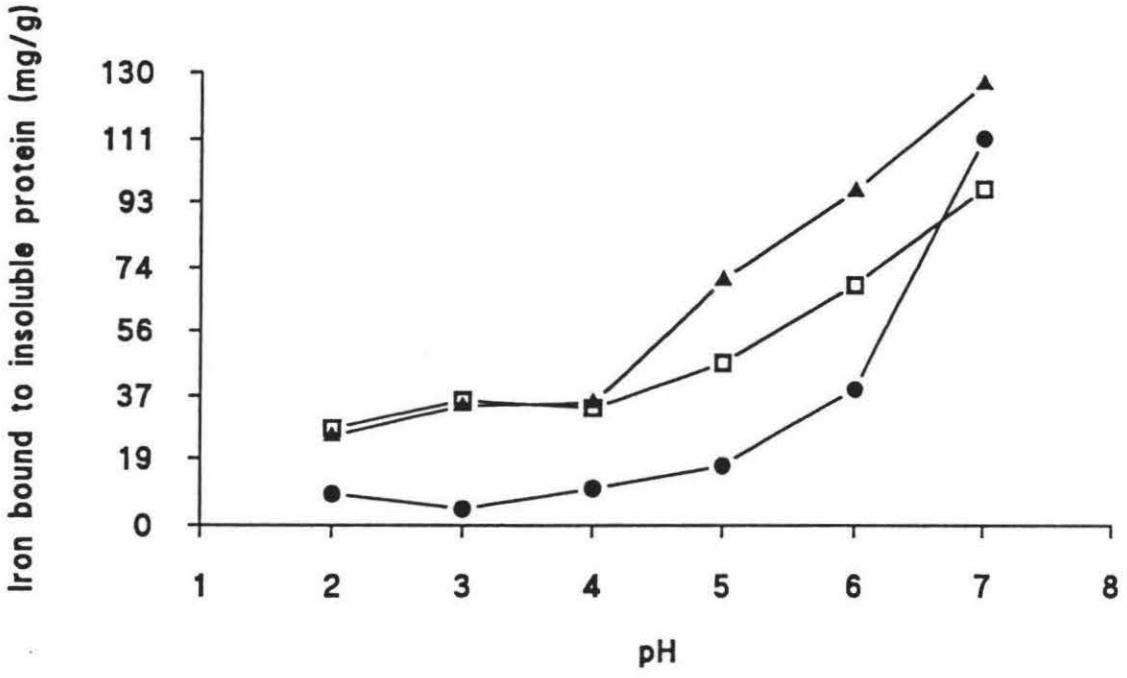


Fig 6.6 (c). Binding of iron to the insoluble fraction of SPI in the absence of acids (●) or in the presence of citric acid (□) and ascorbic acid (▲) at different pH values

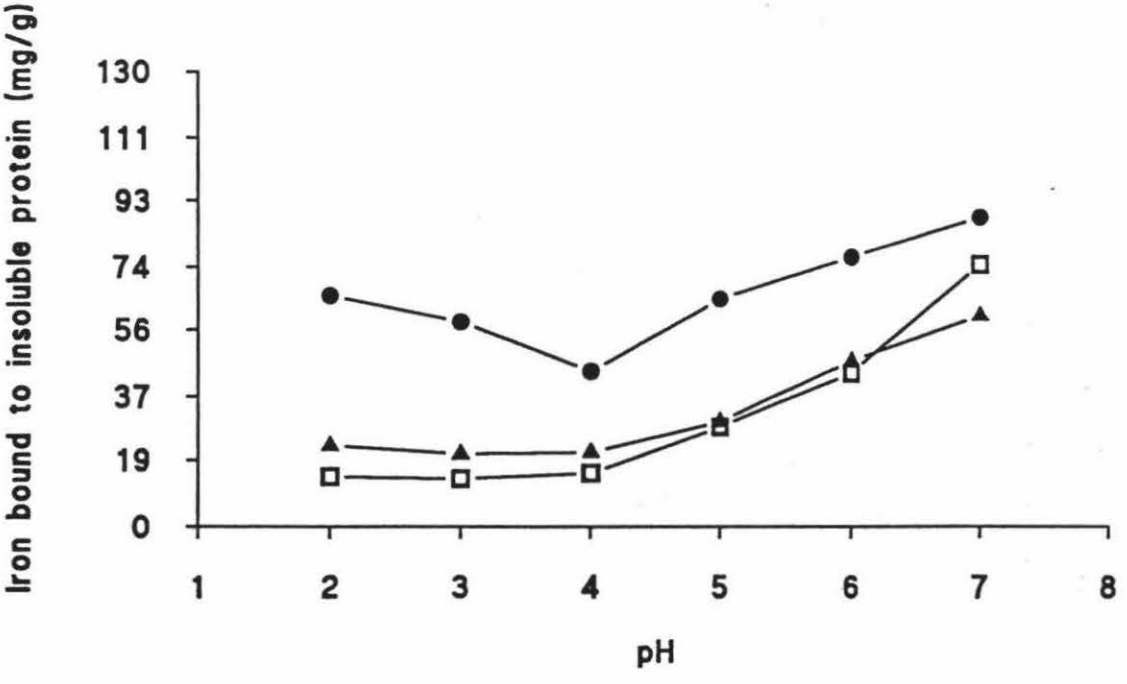


Fig 6.6 (d). Binding of iron to the insoluble fraction of albumen in the absence of acids (●) or in the presence of citric acid (□) and ascorbic acid (▲) at different pH values

citric and ascorbic acid between pHs 2 and 4; beyond pH 5, significantly more iron was bound in the presence of ascorbic acid (Fig. 6.6c).

There was a significant decrease in the amount of iron bound by the insoluble fraction of egg albumen on addition of citric and ascorbic acid (Fig. 6.6d). Binding, in the absence of acid, decreased between pH 2 and 4 but further increase in pH led to gradual increase in binding. In the presence of acid, there was no significant difference in the amounts of iron bound when the pH was increased from 2 to 4 but there was a steady increase in binding between pH 5 and 7. The insoluble fraction of egg albumen essentially bound similar amounts of iron in the presence of citric acid or ascorbic acid (Fig. 6.6d).

6.5.2. Binding to the soluble fraction of the proteins

Addition of ascorbic acid and citric acid decreased the binding of iron by the soluble fraction of WPI between pH 2 and 5, but at pHs 6 and 7, there was a slight increase in binding in the presence of ascorbic and citric acid (Fig 6.7a). Binding by the soluble fraction of WPI in the presence of citric acid and ascorbic acid did not vary significantly with change in pH.

Binding of iron by the soluble fraction of casein decreased significantly on addition of ascorbic and citric acid. In the presence of acids, there was no significant difference in the binding with change in pH (Fig. 6.7b). Casein bound essentially similar amounts of iron in the presence of citric and ascorbic acid, except at pH 6 and 7; at these pHs, the soluble fraction of casein bound slightly less iron in the presence of ascorbic acid.

Addition of citric acid and ascorbic acid decreased the binding by the soluble fraction of SPI, except in the presence of ascorbic acid at pH 7. SPI bound 11 mg iron/g protein which increased to 41 mg/g protein in the presence of ascorbic acid at pH 7 (Fig. 6.7c). Binding in the presence of acids did not follow any consistent pattern. Regardless of the pH, SPI bound greater amounts of iron in the presence of citric acid than in the presence of ascorbic

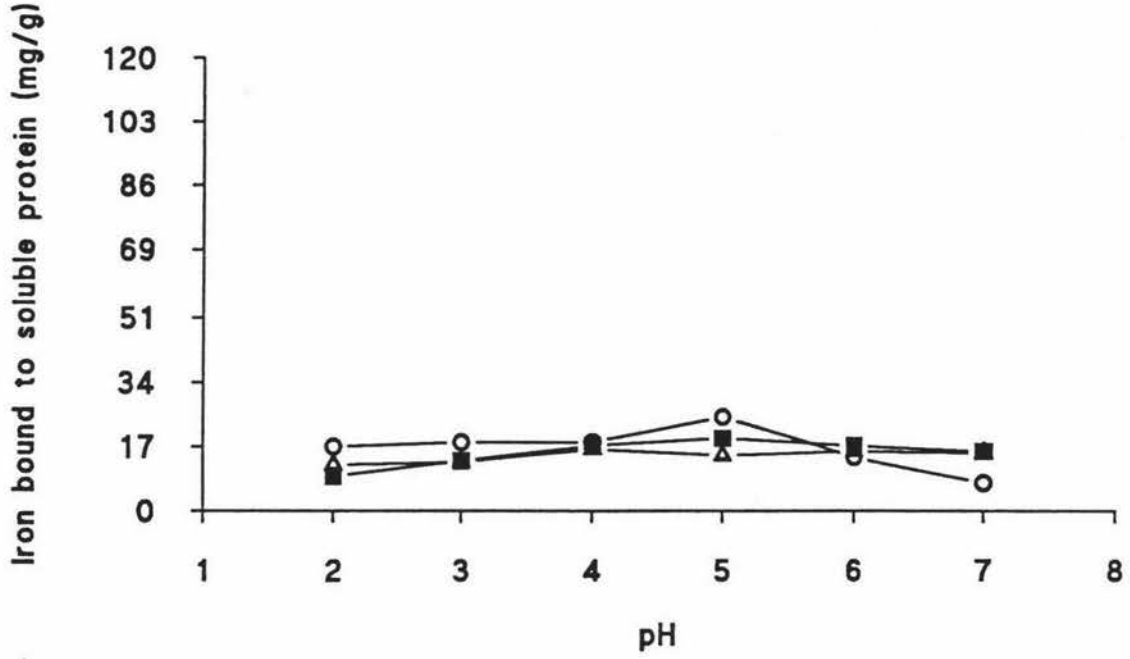


Fig 6.7 (a). Binding of iron to the soluble fraction of WPI in the absence of acids (o) or in the presence of citric acid (■) and ascorbic acid (Δ) at different pH values

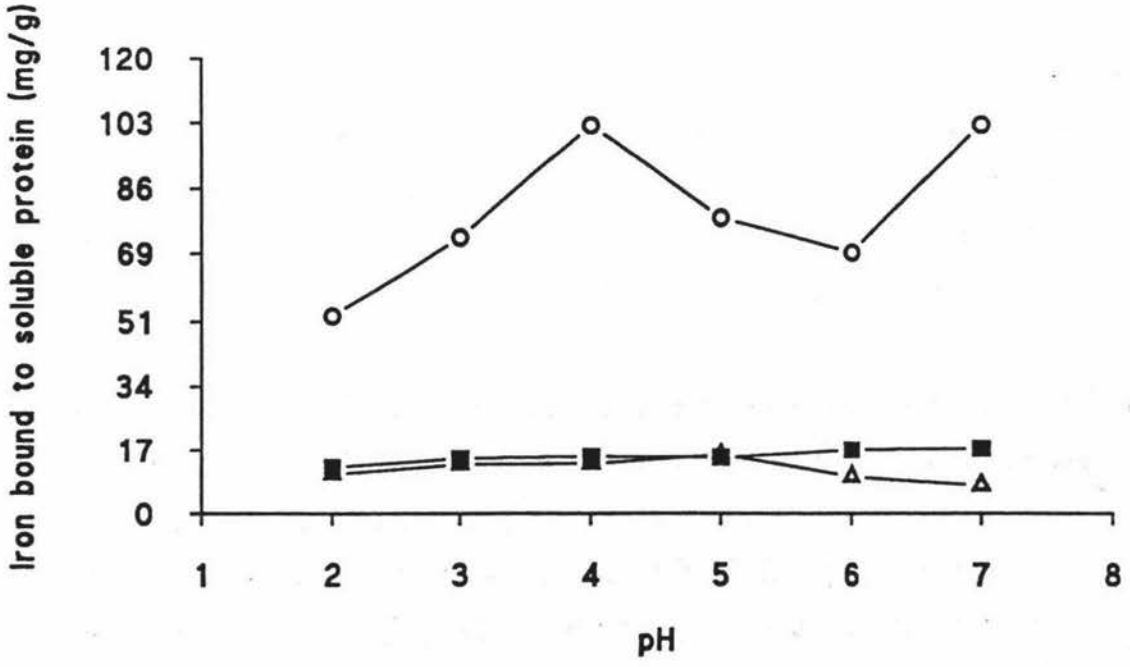


Fig 6.7 (b). Binding of iron to the soluble fraction casein in the absence of acids (o) or in the presence of citric acid (■) and ascorbic acid (Δ) at different pH values

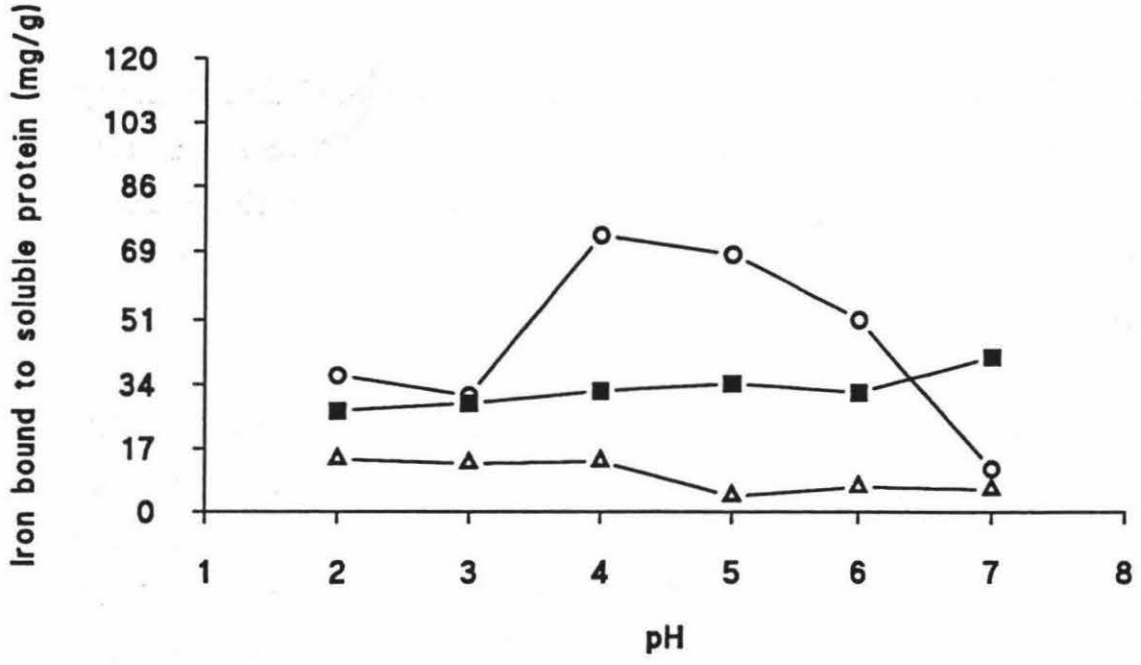


Fig 6.7 (c). Binding of iron to the soluble fraction of SPI in the absence of of acids (o) or in the presence of citric acid (■) ascorbic acid (Δ) at different pH values

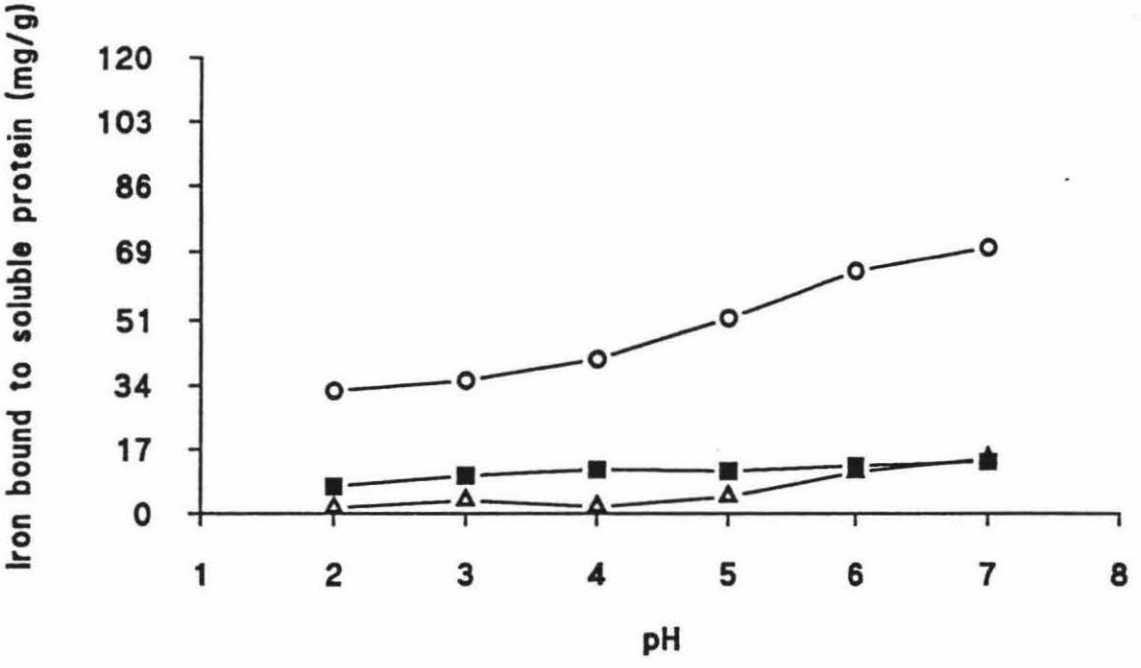


Fig 6.7 (d). Binding of iron to the soluble fraction of albumen in the absence of acids (o) or in the presence of citric acid (■), and ascorbic acid (Δ) at different pH values.

acid (Fig.6.7c).

Addition of citric acid and ascorbic acid significantly decreased the binding by the soluble fraction of albumen (Fig. 6.7d). Between pH 2 and 5, greater decrease in binding was in the presence of ascorbic acid; at pHs 6 and 7 albumen bound essentially similar amounts of iron in the presence of citric and ascorbic acid (Fig. 6.7d).

The solubilizing effects of ascorbic acid and citric acid on iron associated with proteins was dependent on the pH and also protein source. In the presence of acids there was increase in binding with raise in pH which is similar to the findings reported by Kojima *et al* (1981) and Rizk and Clydesdale (1983). Similar findings were reported by Nojiem and Clydesdale (1981) and Gorman and Clydesdale (1983), in the presence of ascorbic acid.

CHAPTER 7

IN VITRO ESTIMATION OF AVAILABILITY OF IRON BOUND TO WHEY PROTEIN, CASEIN, SOYA PROTEIN AND EGG ALBUMEN

Evaluation of diets for iron adequacy requires knowledge of both the amount and availability of iron present (Monsen *et al.*, 1978). While information of iron content of foods is reasonably adequate, knowledge of food iron availability is incomplete. According to Miller and Schricker (1982) this gap in our understanding of the potential of dietary iron to meet nutritional needs exists because a number of complex and interacting factors influence food iron availability. Earlier studies (Nelson and Potter, 1979; Miller and Schricker, 1982) have shown that proteins effect iron absorption.

Iron binding studies showed that iron is bound to both soluble and insoluble fractions of WPI, casein, SPI and egg albumen. *In vivo* formation of protein-iron complex may occur as a result of ingestion of protein and iron simultaneously in a meal or protein-iron complexes may eventually find use in fortification where free iron salts are too reactive (Nelson and Potter, 1979). In either case, the bio-availability of iron from such complexes is of interest.

In this study the method used for estimation of *in vitro* availability involves simulated gastrointestinal digestion followed by measurement of soluble iron. The amount of iron released after pancreatin digestion gives an indication of iron which is free, hence available.

7.1. Enzymatic release of bound iron

The results of pH and enzyme (pepsin and pancreatin) treatment on iron release *i.e.* the low molecular weight soluble iron that pass through the ultrafiltration membrane, from four proteins are shown in Fig 7.1. The percentage of iron released differed significantly for each protein source and was affected by pH and the enzyme treatment.

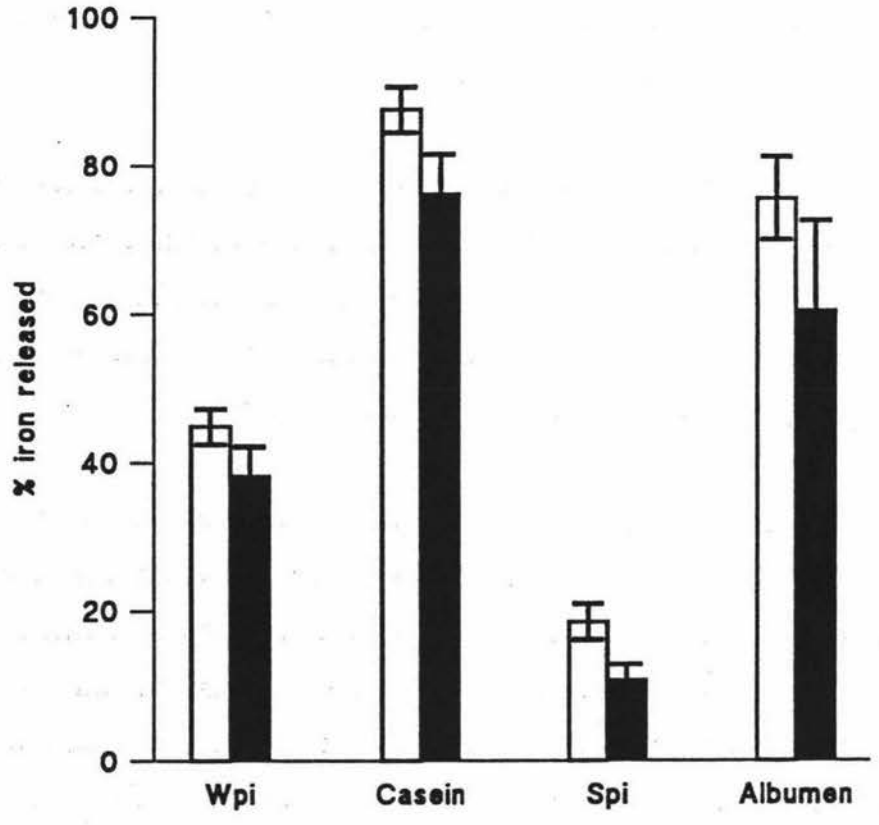


Fig 7.1. % iron released at pH 1.6 after 2h incubation, (□) with enzyme and (■) without enzyme.

In samples adjusted to pH 1.6 with no added pepsin, the percent iron released was lower than the corresponding samples with added pepsin (Fig.7.1). The percentage of iron released after pepsin action (pH 1.6) was greatest for casein (87%) followed by albumen (75%), WPI (44%) and SPI (18%).

Subjecting the pepsin digest to a further enzymatic digestion at pH 7.0 by addition of pancreatin led to a decrease in the percentage of iron released, except in the case of iron bound to SPI (Fig. 7.2). When comparison was made between percent iron released from protein-iron mixture incubated with and without enzyme, more iron was released in the presence of the enzyme. After pancreatin digestion, the release of iron followed the order SPI (26%) > casein (16.1%) > albumen (14%) > WPI (10%) (Fig 7.2).

In the absence of the enzyme, the percentage of iron released indicates the effect of pH; the decrease in iron released with increase in pH agrees well with the findings of previous researchers (Narasinga Rao and Prabhavathi, 1978; Miller and Schricker, 1983). At pH 7, iron forms insoluble iron hydroxides which may explain the decrease in iron released.

The increase in iron release on addition of pepsin agrees well with the findings of Nelson and Potter (1979). The same authors reported that there was further increase in the quantity of iron released with addition of pancreatin, but in the present study there was a decrease in the amount of iron released upon addition of pancreatin, except in the case of iron bound to SPI. Nelson and Potter (1979) suggested that iron release parallels protein digestion, but the results of the present study indicate that iron release was not solely dependent on protein digestibility. Although addition of pancreatin led to increased breakdown of protein, there was a decrease in iron release compared to iron released after pepsin digestion. This suggests that other factors, such as pH and the solubility of protein digestion products may be important in determining the extent of release of bound iron. Enzymatic digestion of protein leads to the release bound iron, but at pH 7.0 (pH of the

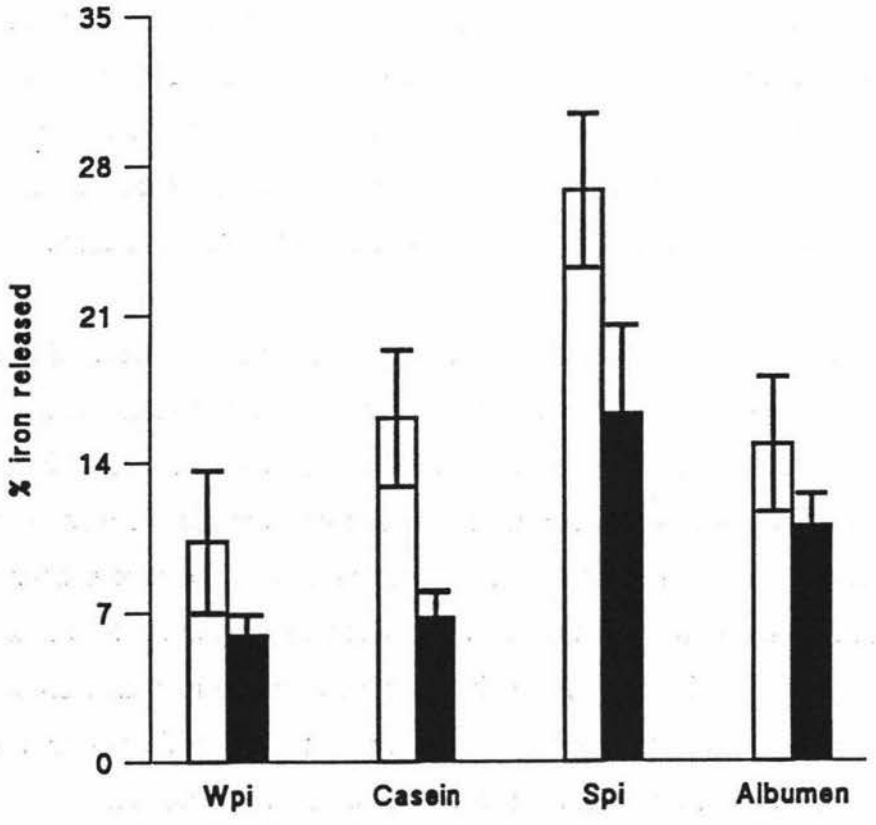


Fig 7.2. % iron released at pH 7.0, after 4h incubation, (□) with enzyme and (■) with out enzyme.

protein-iron mixture) iron forms insoluble hydroxides, the bound iron rendered free due to the digestion of protein might have precipitated, this may explain the decrease in the amount of free iron after pancreatin digestion.

The biologically available iron is the iron released after pancreatin digestion, although several researchers (Jacobs and Greenman, 1969; Ranhotra *et al.*, 1971) have suggested that iron extractable from foods after acid and pepsin digestion could be used as a measure of iron availability; this is not a valid approach as indicated by Fig 7.1. Iron became soluble when the pH was dropped to 1.6 with HCl, and addition of pepsin led to a further increase in soluble iron, but with increase in pH, even after addition of pancreatin, the quantity of soluble or low molecular weight iron remained low. This suggests that if acid and pepsin digestion was taken as the end point of *in vitro* availability, it would lead to an over estimation of the available iron.

In the case of WPI and casein, only a small amount (approximately 6.5%) of added iron was released or became soluble after pancreatin digestion (Fig. 7.2). This indicates that iron bound to milk proteins is not made easily available, which agrees well with the findings of previous researchers. Peters *et al.* (1971) found that in the presence of milk, there was a marked decrease in the dialysability of iron. In two balance studies (Sharpe *et al.*, 1950; Abernathy *et al.*, 1965) milk depressed iron absorption from ferric chloride. Similar observations were also made by Cook and Monsen (1976) who found a decrease in iron absorption in the presence of milk and cheese.

SPI released 16% of bound iron upon pancreatin digestion. Although the quantity of iron released was higher than other proteins, the low value obtained suggests that iron added to SPI was not easily absorbable. Results of previous studies show varying effects of soya proteins on the availability of iron, some showing a depression of iron absorption (Cook *et al.*, 1981), and others showing no effect (Young and Janghorbani, 1981), or even an

enhancement (Ahsworth *et al*, 1973). Schicker *et al.* (1982) found a negative correlation between the protein content of a number of soy protein products and percentage dialysability of iron.

Enzymatic digestion of egg albumen led to a release of 11% of bound iron. Cook and Monsen (1979) found that there was a decreased iron absorption from a semi-synthetic meal in the presence of isolated egg albumen. Although they used purified product as used in the present study they suggested that the inhibitory effect of this protein was related to components other than protein like calcium and phosphorus contained in egg albumen. The quantities of calcium and phosphorus are low in egg albumen, and later studies (1980) by the same authors showed that the addition of calcium and phosphorus in similar amounts as present in purified egg albumen, to a mixture of amino acids, showed no inhibitory effect.

The varying quantities of iron released from different proteins (Figure 7.1 and 7.2) after pepsin and pancreatin digestion may be due to differences in the rate of protein degradation (Cook and Monsen, 1979) and the affinity of these digestion products for iron (Berner and Miller, 1985). If the time of incubation and enzyme concentration is sufficient to degrade protein to low molecular weight peptides, iron release was enhanced. These products maintain iron in a low molecular weight form and prevent the formation of insoluble hydroxide at pH 7.

The amino acid composition of the protein also plays a significant part in determining the quantity of iron released. Studies by Layrisse *et al.* (1974) showed that cysteine had an enhancing effect on iron absorption. Histidine and glutamine were shown to have marked influence on iron availability (Kroe *et al.*, 1966; van Campen and Gross, 1969). The variation in the amino acid composition of the proteins might account for the differences in the percentage of iron released in the presence of different proteins.

7.2. Effects of citric acid and ascorbic acid on enzymatic release of iron bound to protein

Addition of citric acid and ascorbic acid led to an increase in the quantity of iron released from all four proteins (Figs 7.3 and 7.4).

Approximately 55% of the iron bound to WPI was released after digestion with pepsin (pH 1.6), in the presence of citric and ascorbic acid, a 10% increase over the percentage of iron released in the absence of chelating agents (Fig 7.3). After the addition of pancreatin (pH 7.0) and a further 2h incubation, ~23% of bound iron was released in the presence of citric acid and ascorbic acid; this is twice as much as the iron released in the absence of chelating agents (Fig. 7.4). Overall, the results indicate that citric acid and ascorbic acid had similar effects on the release of iron bound to WPI.

About 87% of iron bound to casein was released at the end of pepsin digestion (pH 1.6), which increased to 92% and 90% upon addition of citric acid and ascorbic acid, respectively (Fig. 7.3). After pancreatin digestion (pH 7.0), 16% of bound iron was released which increased to 31% in the presence of citric acid and 33% in the presence of ascorbic acid (Fig. 7.4).

There was an increase in the quantity of iron released when citric acid and ascorbic acid were added to SPI-iron mixture. Analysis of the filtrate of pepsin digest showed that 18% of bound iron was released but when citric acid or ascorbic acid was added prior to digestion with pepsin, the percentage of iron released increased almost two-fold (32%) (Fig. 7.3). After pancreatin treatment, 26% of bound iron was released which increased to 63% and 57% in the presence of citric acid and ascorbic acid, respectively (Fig. 7.4).

When albumen-iron mixture was subjected to pepsin treatment (pH 1.6), 75% of bound iron was released, which increased to 88% upon addition of citric acid and 80% upon addition of ascorbic acid (Fig 7.3). After pancreatin digestion (pH 7.0), ~15% of bound iron was released when digested with

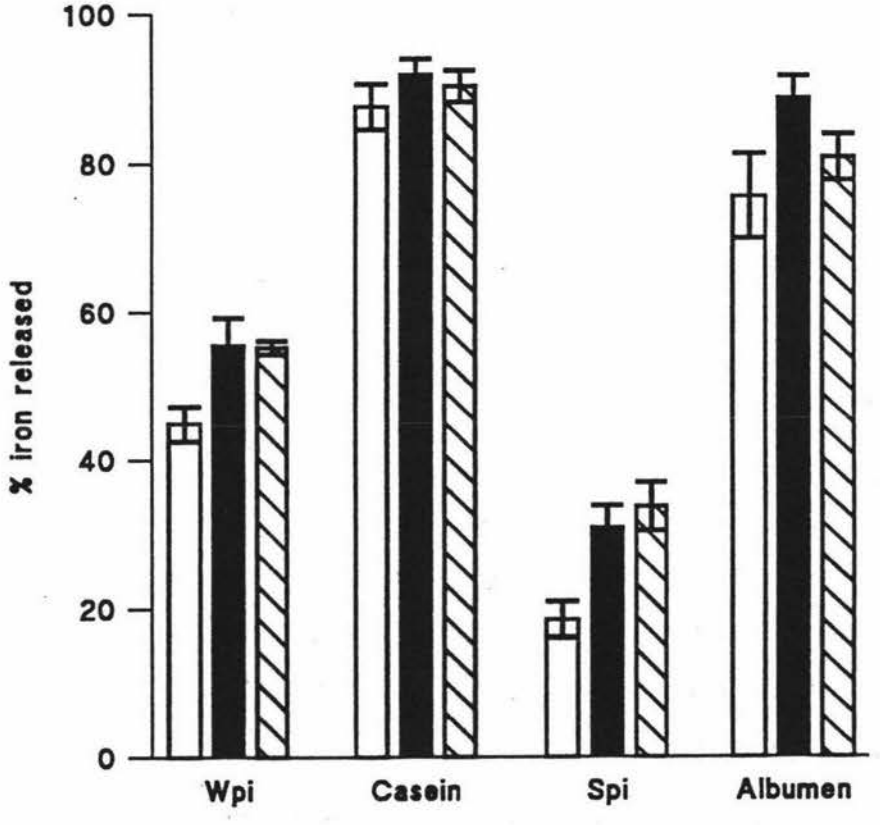


Fig 7.3. % iron released after pepsin digestion (pH 1.6), without acid (□), with citric acid (■) and with ascorbic acid (▨).

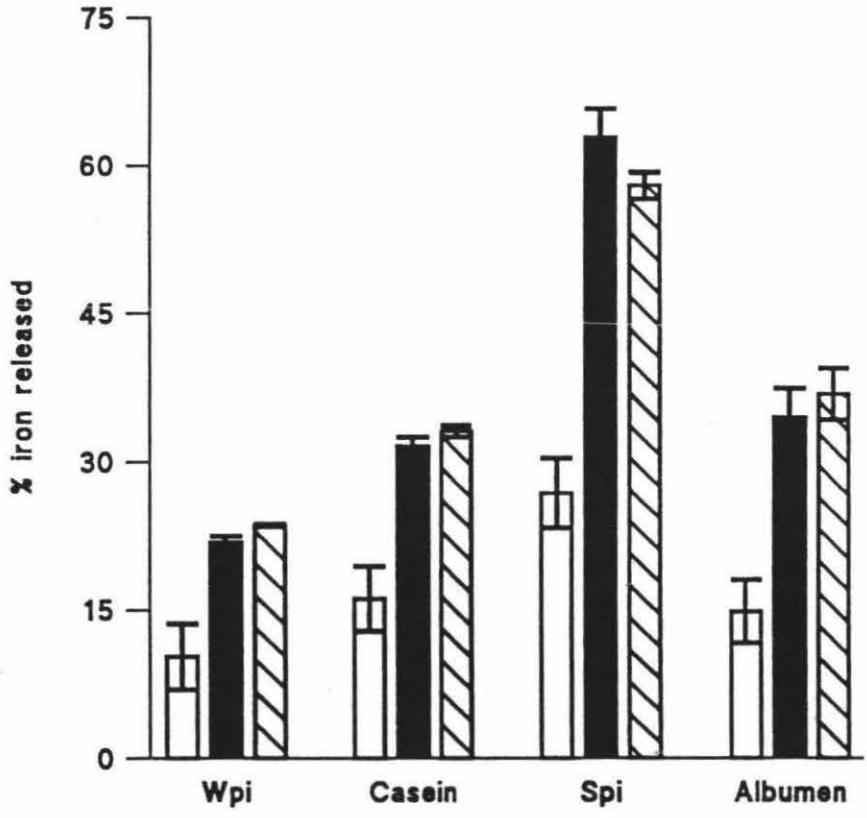


Fig 7.4. % iron released after pancreatin digestion (pH 7.0), without acid (□) with citric acid (■) and with ascorbic acid (▨).

pancreatin in the absence of chelating agents. Addition of citric acid and ascorbic acid caused a significant increase in the release of bound iron. In the presence of citric acid, 34% of bound iron was released; when ascorbic acid was added in similar amounts it was observed that 36% of bound iron was released (Fig. 7.4).

It is clear from the result that addition of citric acid and ascorbic acid, followed by pepsin digestion (pH 1.6) had greatest effect on the release of iron bound to SPI, followed by albumen, WPI and casein. The trend differed slightly after pancreatin digestion (pH 7.0), the greatest effect was on iron bound to SPI, followed by albumen, casein and WPI. There was no significant differences between the effects of citric acid and ascorbic acid.

The increase in the quantity of bound iron released by the addition of citric acid and ascorbic acid indicates that these two chelating agents are likely to enhance the absorption of iron. This finding agrees well with that of several researchers who demonstrated the enhancing effect of citric acid and ascorbic acid on iron absorption (Callender *et al.*, 1970; El- Hawary *et al.*, 1974, Cook *et al.*, 1977; Gillooly *et al.*, 1984). Hallberg (1976) suggested that the enhancing effect of ascorbic acid and citric acid on iron absorption may be related to both its reducing effect, preventing the formation of insoluble ferric hydroxides, and its effect of forming soluble complexes with iron, which preserve iron solubility in the more alkaline duodenal pH (7). Addition of ascorbic acid and citric acid had greater effect on release of bound iron, after pancreatin digestion than after pepsin digestion. Low pH and pepsin action favour the release of bound iron even in the absence of chelating agents, hence, the effect of chelating agents was not pronounced. But a significant effect, after pancreatin digestion, suggests that bound iron which was rendered free upon pancreatin digestion formed soluble complexes with ascorbic acid and citric acid, and this complex formation prevented the precipitation of iron by preventing the formation of insoluble hydroxide. In the absence of chelating agents, the iron which was released from the protein

upon protein degradation and iron solubilized by low pH (1.6) would form insoluble hydroxides at higher pH (7.0) leading to a lesser quantity of low molecular weight absorbable iron.

CONCLUSIONS

From the series of investigations the following conclusions can be drawn.

* From the four food proteins studied iron bound/g protein was highest for casein followed by albumen, soya protein and whey protein. Binding by both soluble and insoluble protein fractions showed similar trend.

* Soya protein has maximum number of binding sites for iron (203) and whey protein binds iron more strongly.

* The amount and strength of binding of iron to protein is dependent on the pH of the protein-iron mixture.

* Chelating agents solubilize protein and iron by decreasing association of iron and protein, by forming low molecular weight chelates with iron.

* The solubilizing effect of the chelating agents studied is dependent on the protein source and pH.

* There was no significant difference in the solubilizing effects of the two acids (Citric acid and ascorbic acid).

* Enzymatic digestion of protein, with HCl-pepsin and pancreatin resulted in release of bound iron, with highest iron release by soya protein followed by casein, albumen and whey protein.

* Addition of citric acid and ascorbic acid prior to enzymatic digestion increased bioavailability.

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