

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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

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

**NETRA MANVIKAR**

**1993**

### 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 soya 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).

### ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. Harjinder Singh and Dr. Juliet Wiseman, for their guidance, advice and continued encouragement during the course of this investigation.

I would also like to thank all the staff of the Department of Food Technology, especially the following:

Mr Hank Van Til, Mrs Margaret Bewley, Mr Steve Glasgow, and Mr Alistair Young for their technical assistance.

Mrs Lesely James, Ms June Latham and Miss Rebecca Baxter for their encouragement and help during the course of this work.

I am very grateful to Mr Ranjan Sharma, Ms Janet Weber and Mr Tang Qingnong for their valuable advice and help.

I would also like to thank all my fellow graduate students, particularly Gayathri, Wibha, Pinthita, David and Sarathy for their encouragement, assistance and friendship.

I would like to extend my thanks to Rani, Bente, Marijke, Claudia and Joost for their friendship and help without which my stay here would not have been so enjoyable.

Special thanks is extended to my 'Hosts' - Mr and Mrs Lakshmanan, and other friends in Palmerston North for making my stay in a foreign land comfortable.

Finally, my greatest appreciation is extended to my sister Mrs Veda Sattur and her husband Mr Avinash Sattur, and my family for their continued moral support and encouragement and this thesis is dedicated to them.

## TABLE OF CONTENTS

<b>ABSTRACT</b>	I
<b>ACKNOWLEDGMENTS</b>	III
<b>TABLE OF CONTENTS</b>	IV
<b>LIST OF FIGURES</b>	VIII
<b>LIST OF TABLES</b>	XVI
<b>CHAPTER 1. INTRODUCTION</b>	1
<b>CHAPTER 2. REVIEW OF LITERATURE</b>	3
2.1. Chemistry of proteins	3
2.1.1. Whey proteins	4
2.1.1.1. Manufacture of WPI's and WPC's	5
2.1.2. Caseins	6
2.1.2.1. Manufacture of caseinate	7
2.1.3. Soya proteins	8
2.1.3.1. Manufacture of isolated soya proteins	8
2.1.5. Egg albumen	9
2.1.5.1. Separation of egg white proteins	11
2.2. Binding of proteins to metal ions	12
2.2.1. Determining binding sites and binding constants	13
2.3. Interactions of iron with food proteins	14
2.3.1. Milk proteins	14
2.3.2. Soya proteins	16
2.3.3. Egg proteins	17
2.4. Effect of chelating agents on the binding of iron to food proteins	19
2.4.1. EDTA	19
2.4.2. Ascorbic acid	19
2.4.3. Citric acid	20
2.5. Measurement of bioavailability	21
2.5.1. Variables to be controlled in <i>in vitro</i> studies	22
2.5.1.1. pH	23
2.5.1.2. Concentration of digestive enzymes	23

2.5.1.3. Digestion time	24
2.5.2. Procedures for <i>in vitro</i> studies	24
2.6. Effect of solubility on bioavailability of iron	25
2.6.1. Valency and charge density	25
2.6.2. Particle size	26
2.6.3. pH	26
2.6.4. Dietary components	26
2.7. Role of protein in the absorption of iron	27
2.7.1. Protein digestion	27
2.7.1.1. Gastric digestion	27
2.7.1.2. Proteolysis in the intestine	27
2.7.2. Mechanism of iron absorption	27
2.7.3. Role of amino acids and peptides	28
2.8. Nutritional relevance of protein iron interaction	29
2.9. Summary	30
<b>CHAPTER 3. OBJECTIVES</b>	<b>32</b>
<b>CHAPTER 4. MATERIALS AND METHODS</b>	<b>33</b>
4.1. Materials	33
4.1.1. Iron source	33
4.1.2. Protein sources	33
4.2. Reagents	33
4.2.1. Hepes buffer	33
4.2.2. Ascorbic acid solution	33
4.2.3. Citric acid solution	34
4.2.4. Enzyme solutions	34
4.3. Analytical methods	34
4.3.1. Iron concentration	34
4.3.1.1. Acid digestion procedure	34
4.3.2. Protein concentration	34
4.4. Experimental procedure	35

4.4.1. Solubility of ferrous sulphate	35
4.4.2. Iron binding studies	35
4.4.3. Effect of pH on iron binding	36
4.4.4. Effect of chelating agents on iron binding	36
4.4.5. In vitro availability studies	36
4.4.5.1. Protein iron mixtures	36
4.4.5.2. Pepsin digestion	36
4.4.5.3. Pancreatin digestion	37
4.4.5.4. Modified procedure for iron availability studies	38
4.4.5.5. Effect of chelating agents on iron release	39

## **CHAPTER 5. BINDING OF IRON TO WHEY PROTEIN, CASEIN, SOYA PROTEIN AND EGG ALBUMEN**

5.1. Experimental design	40
5.2. Effect of iron concentration on protein solubility	41
5.3. Binding of iron to insoluble protein fraction	41
5.4. Binding of iron to soluble protein fraction	46
5.4.1. Binding sites and binding constants	50
5.5. Effect of pH on the solubility of protein and iron	54
5.6. Effect of pH on the binding of iron to protein	58

## **CHAPTER 6. EFFECT OF ADDITION OF CHELATING AGENTS ON THE BINDING OF IRON TO WHEY PROTEIN, CASEIN, SOYA PROTEIN AND EGG ALBUMEN**

6.1. Effect of addition of chelating agents on the solubility of protein and iron	63
6.2. Effect of addition of chelating on the binding of iron to proteins	69
6.2.1. Binding to the insoluble fraction	69
6.2.2. Binding to the soluble fraction	71
6.3. Effect of chelating agents on the solubility of proteins at different pH values	74



6.4. Effect of chelating agents on the solubility of iron at different pH values	77
6.5. Effect of chelating agents on the binding of iron to the proteins at different pH values	81
6.5.1. Binding to the insoluble fraction of the proteins	81
6.5.2. Binding to the soluble fraction of the proteins	84
<b>CHAPTER 7. BIOLOGICAL AVAILABILITY OF IRON BOUND TO WHEY PROTEIN, CASEIN, SOYA PROTEIN AND EGG ALBUMEN</b>	88
7.1. Enzymatic release of bound iron	88
7.2. Effects of citric and ascorbic acid on the enzymatic release of iron bound to protein	94
<b>CHAPTER 8. CONCLUSIONS</b>	99
<b>BIBLIOGRAPHY</b>	100

## LIST OF FIGURES

Figure		Page
2.1	Absorption of nonheme iron from semisynthetic meals. Absorption ratios compare % iron absorption of non heme iron from test meal to % absorption of nonheme iron from semisynthetic meals.	18
5.1	Experimental procedure for determining the binding of iron to proteins.	40
5.2 (a)	Influence of iron concentration on the solubility of WPI (▲) and iron (○) in 10 mM Hepes buffer at pH 6.6.	42
5.2 (b)	Influence of iron concentration on the solubility of casein (▲) and iron (○) in 10 mM Hepes buffer at pH 6.6.	42
5.2 (c)	Influence of iron concentration on the solubility of SPI (▲) and iron (○) in 10 mM Hepes buffer at pH 6.6.	43
5.2 (d)	Influence of iron concentration on the solubility of egg albumen (▲) and iron (○) in 10 mM Hepes buffer at pH 6.6.	43
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.	44
5.3 (b)	Influence of iron concentration on the binding of iron to the insoluble fraction of albumen in 10 mM Hepes buffer at pH 6.6.	44

<b>Figure</b>		<b>Page</b>
5.3. (c)	Influence of iron concentration on the binding of iron to the insoluble fraction of WPI in 10 mM Hepes buffer at pH 6.6.	45
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.	45
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.	47
5.4 (b)	Influence of iron concentration on the binding of iron to the soluble fraction of albumen in 10 mM Hepes buffer at pH 6.6.	47
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.	48
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.	48
5.5 (a)	Scatchard plot for the binding of iron to WPI.	51
5.5 (b)	Scatchard plot for the binding of iron to casein.	51
5.5 (c)	Scatchard plot for the binding of iron to SPI.	52

<b>Figure</b>		<b>Page</b>
5.5 (d)	Scatchard plot for the binding of iron to egg albumen.	52
5.6 (a)	Effect of pH on the solubility of WPI and iron at protein : iron ratio of 1:0.1. (○) protein with iron, (□) protein without iron and (▼) iron.	55
5.6 (b)	Effect of pH on the solubility of casein and iron, at protein : iron ratio of 1 : 0.16. (○) protein with iron, (□) protein without iron and (▼) iron.	55
5.6 (c)	Effect of pH on the solubility of SPI and iron at protein : iron ratio of 1 : 0.16. (○) protein with iron, (□) protein without iron and (▼) iron.	56
5.6 (d)	Effect of pH on the solubility of albumen and iron at protein : iron ratio of 1 : 0.18. (○) protein with iron (□) protein without iron and (▼) iron.	56
5.7 (a)	Effect of pH on the binding of iron to the soluble (○) and insoluble (▲) fractions of WPI at 100 mg iron/g protein.	59
5.7 (b)	Effect of pH on the binding of iron to the soluble (○) and insoluble (▲) fractions of casein at 160 mg iron/g protein.	59
5.7 (c)	Effect of pH on the binding of iron to the soluble (○) and insoluble (▲) fractions of SPI at 160 mg iron/g protein.	60

**Figure****Page**

- 5.7 (d) Effect of pH on the binding of iron to the soluble (○) and insoluble (▲) fractions of egg albumen at 180 mg iron/g protein. 60
- 6.1 (a) Effect of addition of ascorbic acid and 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 (□). 64
- 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 (□). 64
- 6.1 (c) Effect 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 (□). 65
- 6.1 (d) Effect of addition of ascorbic acid and 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 (□). 65
- 6.2 (a) Solubility of iron in WPI-iron mixture, (Δ) with citric acid and (▲) with ascorbic acid. 67
- 6.2 (b) Solubility of iron in casein-iron mixture, (Δ) with citric acid and (▲) with ascorbic acid. 67

<b>Figure</b>		<b>Page</b>
6.2 (c)	Solubility of iron in SPI-iron mixture, ( $\Delta$ ) with citric acid and ( $\blacktriangle$ ) with ascorbic acid.	68
6.2 (d)	Solubility of iron in albumen-iron mixture, ( $\Delta$ ) with citric acid and ( $\blacktriangle$ ) with ascorbic acid.	68
6.3 (a)	Effect of addition of ascorbic acid ( $\circ$ , $\square$ ), and citric acid ( $\bullet$ , $\blacksquare$ ) on the binding of iron to soluble ( $\square$ , $\blacksquare$ ) and insoluble ( $\circ$ , $\bullet$ ) fractions of WPI.	70
6.3 (b)	Effect of addition of ascorbic acid ( $\circ$ , $\square$ ), and citric acid ( $\bullet$ , $\blacksquare$ ) on the binding of iron to soluble ( $\square$ , $\blacksquare$ ) and insoluble ( $\circ$ , $\bullet$ ) fractions of casein.	70
6.3 (c)	Effect of addition of ascorbic acid ( $\circ$ , $\square$ ), and citric acid ( $\bullet$ , $\blacksquare$ ) on the binding of iron to soluble ( $\square$ , $\blacksquare$ ) and insoluble ( $\circ$ , $\bullet$ ) fractions of SPI.	72
6.3 (d)	Effect of addition of ascorbic acid ( $\circ$ , $\square$ ), and citric acid ( $\bullet$ , $\blacksquare$ ) on the binding of iron to soluble ( $\square$ , $\blacksquare$ ) and insoluble ( $\circ$ , $\bullet$ ) fractions of egg albumen.	72
6.4 (a)	Solubility of WPI in protein-iron mixture ( $\bullet$ ), protein protein-iron-citric acid ( $\square$ ) and protein-iron ascorbic acid ( $\blacksquare$ ) mixtures at different pH values.	75
6.4 (b)	Solubility of casein in protein-iron mixture ( $\bullet$ ), protein protein-iron-citric acid ( $\square$ ) and protein-iron ascorbic acid ( $\blacksquare$ ) mixtures at different pH values.	75

<b>Figure</b>		<b>Page</b>
6.4 (c)	Solubility of SPI in protein-iron mixture (●), protein protein-iron-citric acid (□) and protein-iron ascorbic acid (■) mixtures at different pH values.	76
6.4 (d)	Solubility of albumen in protein-iron mixture (●), protein protein-iron-citric acid (□) and protein-iron ascorbic acid (■) mixtures at different pH values.	76
6.5 (a)	Effect of pH (○), citric acid (●) and ascorbic acid (□) on the solubility of iron in WPI-iron mixture (100 mg iron and 100 mg acid/g protein).	78
6.5 (b)	Effect of pH (○), citric acid (●) and ascorbic acid (□) on the solubility of iron in casein-iron mixture (160 mg iron and 100 mg acid/g protein).	78
6.5 (c)	Effect of pH (○), citric acid (●) and ascorbic acid (□) on the solubility of iron in SPI-iron mixture (160 mg iron and 100 mg acid/g protein).	79
6.5 (d)	Effect of pH (○), citric acid (●) and ascorbic acid (□) on the solubility of iron in albumen-iron mixture (180 mg iron and 100 mg acid/g protein).	79
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.	82
6.6 (b)	Binding of iron to the insoluble fraction of casein in the absence of acids (●) or in the presence of citric acid (□) and ascorbic acid (▲) at different pH values.	82

<b>Figure</b>		<b>Page</b>
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.	83
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.	83
6.7 (a)	Binding of iron to the soluble fraction of WPI in the absence of acids (○) or in the presence of citric acid (■), and ascorbic acid (▲) at different pH values.	85
6.7 (b)	Binding of iron to the soluble fraction of casein in the absence of acids (○) or in the presence of citric acid (■), and ascorbic acid (▲) at different pH values.	85
6.7 (c)	Binding of iron to the soluble fraction of SPI in the absence of acids (○) or in the presence of citric acid (■), and ascorbic acid (▲) at different pH values.	86
6.7 (d)	Binding of iron to the soluble fraction of albumen in the absence of acids (○) or in the presence of citric acid (■), and ascorbic acid (▲) at different pH values.	86
7.1	% iron released at pH 1.6, after 2h incubation, (□) with enzyme and (■) without enzyme.	89
7.2	% iron released at pH 7.0, after 4h incubation, (□) with enzyme and (■) without enzyme.	91



**Figure****Page**

- 7.3      % iron released after pepsin digestion (pH 1.6), without acid (□), with citric acid (■) and with ascorbic acid (⊠).      95
- 7.4      % iron released after pancreatin digestion (pH 7.0), without acid (□), with citric acid (■) and with ascorbic acid (⊠).      96

## LIST OF TABLES

<b>Table</b>		<b>Page</b>
2.1	Concentrations and properties of proteins in Whey	4
2.2	Composition of Whey Protein Isolate	6
2.3	Composition and properties of Caseins	7
2.4	Composition of Sodium Caseinate	8
2.5	Composition of Isolated Soy protein	9
2.6	Proteins of Egg Albumen	10
5.1	Binding constants and maximum number of binding sites	53