INVESTIGATION OF COMPENSATORY EFFECT OF COPPER MINERAL AND IRON-PROTEIN COMPLEXES ON IRON DEFICIENCY ANEMIA IN HUMAN ENTEROCYTE CELL CULTURE MODEL

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ABSTRACT

INVESTIGATION OF COMPENSATORY EFFECT OF COPPER MINERAL AND IRON-PROTEIN COMPLEXES ON IRON DEFICIENCY ANEMIA IN HUMAN ENTEROCYTE CELL CULTURE MODEL

Iron deficiency is the most encountered worldwide nutritional disease, affecting women, babies, and children. When dietary iron absorption is insufficient to fulfill physiological demands, nutritional iron insufficiency arises. Legumes are a low-cost source of protein that are also beneficial for human health. From a nutritional standpoint, the iron-chelating ability of legume proteins is of importance since they may have high iron mineral stability against *in vitro* digestion because they are bound to proteins. Legume proteins extracts are commonly utilized as functional components; however, their functionality must be proved in a cell culture system by assessing their physiological activity. Furthermore, during iron deprivation disturbances in copper homeostasis have been observed repeatedly in the literature. And this pointed out that copper might have a compensatory effect on anemia caused by iron deficiency dependant cellular signaling mechanisms.

Within this context, the main objectives of this Ph.D. thesis were *(i)* to develop highly bioavailable, edible protein-iron complex hydrolysates from legumes that can be used as an additive in food products and investigate their functional properties against iron deficiency anemia. Also, *(ii)* investigating the compensatory effects of copper mineral on iron deficiency anemia and, *(iii)* its main application for functional food development were other driving forces for the experiments.

It was revealed that protein (peptide)-iron complexes derived from lentil (10:1 ratio) and soybean (20:1 and 40:1 ratios) significantly influenced the iron-dependent gene regulation in enterocyte cells compared to the anemic group. Moreover, intracellular gene regulation was mainly affected by copper treatment in the basolateral side of enterocyte cells during IDA, indicating that blood copper level might have the ability to control the enterocyte iron metabolism at molecular and genetic levels during iron deficiency anemia.

ÖZET

BAKIR MİNERALİNİN VE DEMİR-PROTEİN KOMPLEKSLERİNİN DEMİR EKSİKLİĞİNE BAĞLI ANEMİ ÜZERİNDEKİ İYİLEŞTİRİCİ ETKİLERİNİN İNSAN ENTEROSİT HÜCRE MODELİNDE İNCELENMESİ

Demir eksikliği anemisi, dünya çapında en sık karşılaşılan, kadınları, bebekleri ve çocukları etkileyen besinsel bir eksikliktir. Besinsel demir eksikliği, besinsel demir emiliminin fizyolojik ihtiyaçları karşılamada yetersiz kalması durumunda ortaya çıkar. Baklagiller, insan sağlığı için de faydalı olan düşük maliyetli bir protein kaynağıdır. Baklagil proteinleri demir bağlama yeteneğine sahip olmaları açısından in vitro sindirime karşı yüksek demir stabilitesine sahip olabilirler. Baklagil protein ekstraktları yaygın olarak fonksiyonel bileşenler olarak kullanılmakla birlikte fonksiyonellikleri hücre kültürü sistemlerinde fizyolojik aktiviteleri değerlendirilerek kanıtlanmalıdır. Ayrıca, demir eksikliği sırasında bakır homeostazında görülen dalgalanmalar literatürde sıklıkla belgelenmiştir. Bu da bakır mineralinin demir eksikliğine bağlı hücresel mekanizmaların neden olduğu anemi üzerinde iyileştirici bir etkiye sahip olabileceğini işaret etmektedir.

Bu bağlamda bu doktora çalışmasının temel amaçları; gıda ürünlerinde katkı maddesi olarak kullanılabilecek baklagil türevli yenilebilir ve yüksek biyoyararlanıma sahip protein-demir komplekslerinin geliştirilmesi ve demir eksikliği anemisine karşı bu komplekslerin fonksiyonelliğinin araştırılmasıdır. Ayrıca bakır mineralinin demir eksikliği anemisi üzerindeki telafi edici etkilerinin araştırılması ve fonksiyonel gıda geliştirilmesi için uygulanabilirliği tezin diğer motivasyon kaynaklarını oluşturmaktadır.

Mercimek (10:1 oranında) ve soya fasulyesinden (20:1 ve 40:1 oranında) elde edilen protein (peptit) – demir komplekslerinin anemik gruba kıyasla enterosit hücrelerinde demire bağımlı gen regülasyonunu önemli ölçüde etkilediği ortaya çıkmıştır. Ayrıca, demir eksikliği durumunda enterosit hücrelerinin bazolateral tarafından uygulanan bakır mineralinin hücre içi gen regülasyonunu etkilediği bulunmuştur. Bu da kandaki bakır düzeyinin moleküler ve genetik düzeyde enterosit demir metabolizmasını kontrol etme yeteneğine sahip olabileceğine işaret etmektedir.

This Ph.D. thesis is dedicated to my sweet daughter,

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TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xiv

CHAPTER 1. INTRODUCTION 1

CHAPTER 2. LITERATURE REVIEW 7

2.1. Main Functions of Iron 7

2.1.1. Iron Metabolism and Intestinal Absorption 8

2.1.2. Genetic Regulation of Intestinal Iron Homeostasis 10

2.2. Effects of Copper on Physiological Iron Metabolism 11

2.2.1. Main Functions of Copper 12

2.2.2. Copper Metabolism 12

2.3. Iron in Food 13

2.4. Iron Bioavailability 14

2.5. Importance of Fortification for Iron Deficiency 15

CHAPTER 3. COMPENSATORY RESPONSE OF COPPER MINERAL ON INTESTINAL IRON DEFICIENCY ANEMIA 18

3.1. Cell Culture 19

3.2. Modeling of the Human Intestinal System and Copper Treatments of Apical and Basolateral Sides of the Cells During Iron Deficiency Anemia 20

3.3. Determination of the Effect of Copper on Iron Metabolism at the Molecular and Genetic Level 20

3.4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) 22

3.5. Statistical Analysis 22

3.6. Results and Discussions 23

3.6.1. Modeling of Human Intestine System and TEER Measurements of Monolayer Caco-2 Cells 23

3.6.2. Apical and Basolateral Treatments of Copper in DFO Induced Anemic Monolayer Caco-2 Cells 24
3.6.3. Effect of Copper on Iron Deficiency Anemia in CaCo-2 Cells Grown on the 12-well Tissue Culture Plates 24
3.7. Conclusion 28

CHAPTER 4. INVESTIGATION OF THE EFFECTS OF IRON MINERAL ON IRON METABOLISM UNDER ANEMIC CONDITIONS 30
4.1. Cell Culture 30
4.2. Investigation of Importance of Dietary and Blood Iron on Intestinal Iron Deficiency 31
4.2.1. Determination of Cell Density on 12-well Plates 32
4.2.2. Modeling of Human Intestinal System and Formation of Cell Barrier System 32
4.2.3. Induction of Iron Deficiency and Iron Treatment 34
4.3. Quantitative Real Time Polymerase Chain Reaction 35
4.4. Statistical Analysis 35
4.5. Results and Discussion 37
4.5.1. Caco-2 Cell Line and Effect of Cell Density on Cell Culture 37
4.5.2 Effect of Iron Treatment on Molecular and Genetic Regulation of Iron Metabolism of the Cells Growing on 12-well Plates 38
4.5.3. Effect of Iron Treatment on Molecular and Genetic Regulation of Iron Metabolism of the Cells in Bicameral System (Modeling of Human Intestinal System) 41
4.6. Conclusion 44

CHAPTER 5. FUNCTIONAL PROPERTIES OF LEGUME PROTEINS AND IRON SALT 48
5.1. Materials 48
5.2. Protein Extraction 49

5.3. Total (TPrC) and Water-Soluble Protein Contents (WSPC) of Legume Extracts 50
5.4. Determination of Ferrous Iron Amount and Ferrous Iron Chelating Ability (FIC) of Protein Extracts of Legumes 51
5.5. Determination of Iron Solubility at Various pH Conditions 52
5.6. Statistical Analysis 52
5.7. Results and Discussions 52
5.7.1. Functional Properties of Legumes 52
5.7.1.1. TPrC, WSPC, and pH-dependant Solubility Profile of Lyophilized Legume Protein Extracts and Iron Salt 52
5.7.1.2. Studies on the Determination of Fe^{2+} Chelating Capacity of Lyophilized Crude Protein Extracts 58
5.8. Conclusion 62

CHAPTER 6. FORMATION OF PROTEIN-IRON COMPLEXES AND SIMULATED GASTROINTESTINAL DIGESTION 67
6.1. Formation of Protein-Iron Complexes 68
6.2. Determination of Simulated Gastrointestinal Digestion Assay for Protein Extracts 68
6.2.1. Optimization of Simulated Gastrointestinal Digestion Assay 79
6.3. Enzymatic Hydrolysis Assay for Protein Extracts of Legumes 71
6.4. Statistical Analysis 71
6.5. Results and Discussions 73
6.5.1. Protein and Iron Binding Profiles of Complexes at Physiological pH 73
6.5.2. Simulated Gastrointestinal Digestion Assay for Protein Extracts 79
6.5.2.1. Optimization of Simulated Gastrointestinal Digestion 80
6.5.3. Simulated *in vitro* Digestion Studies of Protein-Iron Complexes 85
6.5.4. Enzymatic Hydrolysis Assay for Protein Extracts of Legumes 94
6.6. Conclusion 95

CHAPTER 7. DETERMINATION OF THE EFFECTS OF PROTEIN-IRON COMPLEXES ON IRON METABOLISM AT THE MOLECULAR AND GENETIC LEVEL 97
7.1. Induction of Iron Deficiency Anemia and Treatment of Digested Protein-Iron Complexes in Caco-2 Cells 98
7.2. Total RNA Isolation and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) 100
7.3. Statistical Analysis 100
7.4. Results and Discussions 101
7.4.1. Cell Studies – Effect of Digested Protein-Iron Complexes on Iron Dependent Gene Regulation 101
7.5. Conclusion 108

CHAPTER 8. CONCLUSION 110

REFERENCES 115

APPENDICES
APPENDIX A. BSA STANDARD CURVE 126
APPENDIX B. Fe^{2+} STANDARD CURVE 127

LIST OF FIGURES

Figure **Page**

Figure 2.1. Iron recycling system in human body 8
Figure 2.2. Iron absorption mechanisms of small intestine enterocyte cells 9
Figure 2.3. Copper absorption mechanisms of small intestine enterocyte cells 13
Figure 3.1. Determination of the effect of copper mineral on the molecular and genetic regulation of iron metabolism in anemic cells growing on 12-well inserts 21
Figure 3.2. Design of the experiments for determination of the effect of copper on iron metabolism at the molecular and genetic level in anemic cells growing on 12-well plates 21
Figure 3.3. Transepithelial electrical resistance (TEER) measurements in the Caco-2 cells grown on bicameral insert for 21 days 23
Figure 3.4. The effect of copper mineral on iron and hypoxia-regulated gene mRNA expression levels under the anemic conditions in polarized Caco-2 cells grown on the bicameral cell culture system for 21 days 25
Figure 3.5. The effect of copper mineral on iron and hypoxia-regulated gene mRNA expression levels under the anemic condition in Caco-2 cells grown on the 12-well cell culture system for 21 days 26
Figure 4.1. Diagram of the investigation experiments of iron mineral on iron metabolism under anemic conditions 31
Figure 4.2. Cell density determination experiment on polystyrene 12-well plates. 32
Figure 4.3. Special bicameral cell culture insert system 33
Figure 4.4. Measurement of the transepithelial electric barrier resistance 33
Figure 4.5. Induction of iron deficiency and subsequent iron treatment in cells growing on 12-well plates and 12-well inserts 34
Figure 5.6. Determination of the effect of iron mineral on the molecular and genetic regulation of iron metabolism in anemic cells growing on 12-well plates. 35

Figure **Page**

Figure 4.7. Determination of the effect of the iron mineral from the diet on polarized anemic cells growing on 12-well inserts 36
Figure 4.8. The effect of iron treatment on iron metabolism on anemic cells growing on 12-well plates. 39
Figure 4.9. The effect of iron treatment on hypoxia-induced gene expression on anemic cells growing on 12-well plates 40
Figure 4.10. 'Melting Curve' results from selected primers 41
Figure 4.11. Determination of cell polarization by TEER and effect of experimental treatments on cell barrier resistance. 42
Figure 4.12. The effect of iron treatment on iron metabolism on anemic cells growing on 12-well inserts 45
Figure 4.13. The effect of iron treatment on hypoxia-induced gene expression on anemic cells growing on 12-well inserts 45
Figure 5.1. Solubility profile of (a) lentil, (b) chickpea, (c) soybean, and (d) pea as g/g protein extract 55
Figure 5.2. Solubility profiles of protein extracts of legumes and iron under different pH conditions 57
Figure 5.3. Na_2-EDTA standard curve study for determination of iron-chelating capacity. 59
Figure 5.4. Iron chelating capacity study for lentil protein extract at physiological pH (pH 7.0). 60
Figure 5.5. Na_2-EDTA standard curves for determination of ferrous ion-chelating capacity. The iron source in the form of a) $FeCl_2$, b) $FeSO_4$. 63
Figure 5.6. Fe^{2+} ion-chelating ability of different lyophilized crude protein extracts. The iron source in the form of a) $FeCl_2$ and b) $FeSO_4$. 64
Figure 5.7. The ferrous ion-chelating capacity of lyophilized crude protein extracts of legumes. 65
Figure 6.1. Diagram of the protein-iron complexation study. 69
Figure 6.2. Flow diagram of the optimized and validated *in vitro* digestion conditions of the legume derived protein-iron complexes. 72
Figure 6.3. Iron binding profiles of legume proteins at different ratios under physiological pH conditions with respect to time 74

Figure **Page**

Figure 6.4. **a)** Soluble protein content and, **b)** Fe^{2+} ion-chelating ability (FIC) of lyophilized lentil protein extract and water as blank after subjecting to different *in vitro* digestion assays 81

Figure 6.5. Effect of termination method on the soluble protein content of samples for *in vitro* gastrointestinal experiments 82

Figure 6.6. One factor plots of a) pepsin concentration, b) pancreatin concentration, c) duration of gastric digestion. d) and e) Response surface plots showing factors and their interactions. 86

Figure 6.7. a) Perturbation plot showing the interaction between enzyme concentrations and duration of gastric phase; b) Numerical optimization; c) Desirability plots. 87

Figure 6.8. The profiles of (A) bioaccessible free iron content (B) soluble protein content of protein–iron complexes derived from legumes at different protein:iron ratios (w/w) after *in vitro* digestion 89

Figure 6.9. a) Soluble protein content of protein-iron complexes b) Bioaccessibility of iron after simulated *in vitro* digestion (Protein-iron complexes formed after digestion) at different concentrations 93

Figure 6.10. Iron-chelating activities of the hydrolysates of lentil, chickpea, soybean, and pea proteins at different hydrolysis times 94

Figure 7.1. Flowchart of design of experiments. 98

Figure 7.2. Induction of iron deficiency and subsequent treatments with iron or protein-iron complexes in cells growing on 12-well plates and 12-well inserts. 99

Figure 7.3. Determination of the effect of iron mineral on the molecular and genetic regulation of iron metabolism in anemic cells growing on 12-well plates 99

Figure 7.4. The effect of protein-iron complexes on iron metabolism and hypoxia-induced gene expression on anemic cells growing on 12-well inserts 103

Figure 7.5. The effects of digested peptide–iron complexes derived from legumes on iron metabolism and hypoxia-induced gene

expression on anemic cells growing on 12-well inserts 106

LIST OF TABLES

Table		Page
Table 3.1.	Marker genes that were used in the evaluation of the effect of copper treatments on iron metabolism	22
Table 4.1.	Marker genes that were used in the evaluation of the effect of treatments on iron metabolism	37
Table 5.1.	Total protein content, water-soluble protein content and ferrous ion-chelating ability protein extracts of legumes	53
Table 6.1.	Box-Behnken design used in the optimization of gastrointestinal digestion assay with respect to the degree of hydrolysis (DH) expressed as soluble protein amount (mg soluble protein per g of protein extract)	83
Table 6.2.	Analysis of variance (ANOVA) for reduced cubic model to determine optimum conditions for simulated gastrointestinal digestion assay	84
Table 7.1.	Marker genes that were used in the evaluation of the effect of treatments on iron metabolism	100

CHAPTER 1

INTRODUCTION

Food Engineering is a field that scientific principles and engineering methods are applied together to generate and sustain a wholesome food supply. Nutrition has a very important place in the studies of food engineering field. Humans and food are discussed in nutrition, as well as the effects of particular food components, foods, or diets on metabolism, the outcomes and causes of under- and overnutrition, and other physiological systems important to the consumer's health. Following breakthroughs in molecular biology and the need to explain the organism's reactions to nutrients at a molecular level, "Molecular Nutrition" has developed as a new field in nutritional researches. It investigates how nutrients, particularly micronutrients, interact with genetic factors to affect human health and the risk of diseases. In other words, molecular nutrition discovers the molecular relationship between food and health.

The main focus of this thesis is iron deficiency which is the most common global nutritional disorder and it is usually tried to be treated with two common approaches as mineral based approach and developing novel dietary supplements. Better understanding of the effects of mineral-mineral interactions on iron deficiency anemia (molecular nutrition part) and development of new dietary supplements based on these mechanisms (food sicence and nutrition part) is important. Considering these two approaches in the treatment of iron deficiency, studies of this Ph.D. thesis were planned under these two main topics.

Chapter 3 and 4 focuses on the effects of mineral-mineral interactions pn iron deficiency. The relationship between copper and iron mineral on iron deficiency anemia was investigated. During iron deficiency, perturbations in copper homeostasis have frequently been documented in the literature. And this pointed out that copper might have a compensatory effect on anemia caused by iron deficiency dependant cellular signaling mechanisms. After obtaining the conclusion that copper mineral plays an important role in iron metabolism in Chapter 3, it was planned another set of experiments in parallel with this study. In the same way, it was designed an experiment to see if the blood iron level by itself can affect iron metabolism in Chapter 4.

The other three chapters (Chapter 5, 6, and 7) focuses on the development of new dietary supplements based on mineral-mineral mechanisms against iron deficiency. It was investigated the effects of protein-iron complexes derived from commonly consumed legumes on iron deficiency anemia in a cell culture system. Legumes are essential for a balanced diet and play a significant role in traditional cuisines across the world. Because of their essential nutrient content, including protein, low glycaemic index carbohydrates, dietary fiber, minerals, and vitamins, they have a high nutritional profile (Çakır et al, 2019). Legume proteins are also known as "poor man's meat" because of their affordable rate compared to animal proteins, as well as their abundance and long-term availability. Protein content in legumes is 2 to 3 times higher than that of cereals, ranging from 17 to 30% in chickpeas, lentils, dry peas, beans and 35-49.6% in soybeans (Sharif et al., 2018). Legume proteins offer functional quality that is important in the food formulation and processing, in addition to their nutritional characteristics (Boye et al., 2010a). Their metal chelating ability is of interest of this thesis.

The American Dietetic Association Pulses has highly recommended pulses and some other legumes to enhance the food quality of the US population (Aydemir and Yemenicioglu, 2013). In addition, the United Nations have proclaimed the year 2016 as the International Year of Legumes/Pulses, recognizing the nutritional value and advantages of legumes (Sharif et al., 2018). Pulses are a type of leguminous crop grown mainly for their dry seed (FAO, 2015) and lentils, dry peas, and chickpeas are the most well-known and consumed types. By definition, soybeans are not considered as pulse because their seed is not dry (it contains high amounts of oil). Chickpea, red lentil, pea, and soybean proteins are of our interest from a nutritional point of view because they may have high stability of iron mineral against *in vitro* digestion since iron minerals are bonded to proteins.

Chickpea (*Cicer arietinum L.*) is an ancient crop that was domesticated in south-eastern Turkey and then it has spread to over 45 nations, making it the world's most widely cultivated crop (Aydemir et al., 2018). India is the world's largest producer of chickpea, followed by Australia, Myanmar, Ethiopia, and Turkey (FAO, 2014). It is a major dietary pulse with rising demand due to its nutritional content, with carbohydrates and high-quality protein accounting for 80% of the chickpea dry seed mass (Monk et al., 2017). Chickpea seeds include a variety of bioactive and functional components that are useful in health-related and other processed food products. The phenolic content of chickpea proteins, hydrolysates, and peptides was high, indicating that they possessed

significant antioxidant activity based on free radical scavenging and metal chelating properties. Chickpea proteins have the potential to be used as functional plant-based protein sources since their functional quality is comparable to or better than those of soy and animal-origin proteins (Aydemir and Yemenicioglu, 2013).

The *Leguminosae* family includes lentils (*Lens culinaris L.*), which are often utilized in traditional diets. Lentils are grown in numerous countries throughout the world. According to the Food and Agriculture Organization of the United Nations Statistical Databases (FAOSTAT), global lentil output was roughly 7.5 million tons in 2017 and between 1994–2017 the top three producers were Canada (1.25 billion), India (950 billion), and Turkey (468 billion). Red and green lentils are the most common types of lentils grown across the globe, however, red lentils account for two-thirds of global output (Roy et al., 2010). Lentils are high in protein as well as other micronutrients. Furthermore, lentils have a protein level of approximately 21%–31% on average, with globulins accounting for 70% of the total protein.

Peas are abundant in protein, dietary fiber, minerals, vitamins and antioxidants, and pea proteins have a high nutritional value due to their amino acid profile, which includes a lot of sulphuric amino acids that are essential for mammals (Mierzejewska et al., 2008).

Soybean (*Glycine max*) is one of the world's most important and commonly consumed legume crops with a global economic effect of $114 billion. The United States and Brazil are the world's major producers of soybean. According to USDA nutritional database, soybean seeds include about 36.5% protein, 19.9% lipids, 30% carbohydrates, and 9.3% dietary fiber. Soybean consumption has risen in recent years as a result of its beneficial impacts on human health. It also serves as a primary source of protein for vegans all over the world (Vagadia et al., 2017).

The link between food and nutrition is critical for sustaining optimal health conditions in humans. Food is now expected to support wellbeing by preventing nutrient-related illnesses, in addition to being a source of important nutrients and a means of alleviating hunger (Admassu et al., 2018). Natural substances have become more popular with this increasing knowledge (Lafarga et al., 2017). As a result of their influence on functional health features such as cell proliferation, inflammation, and metabolic illnesses, peptides of dietary protein are receiving a lot of attention (Chakrabarti et al., 2018). By chelating minerals, certain peptides are engaged in nutrient– nutrient interactions (Eckert et al., 2014) and this might alter cellular mineral metabolism in

humans due to their high stability against *in vitro* digestion or mineral bioavailability in enterocyte cells of the intestine.

Iron is one of the essential trace minerals required for humans, and a lack of it impacts oxygen delivery to tissues, cell development, and energy metabolism (Knutson et al., 2003). Iron deficiency is the most frequent and prevalent worldwide nutritional problem, affecting women, babies and children in particular. It is characterized as a situation in which there are no mobilizable iron reserves and in which evidence of a weakened iron supply to tissues (WHO, 2001). Over 2 billion people, roughly 30% of the world's population, are anemic with iron deficiency being the most common cause. Every second pregnant woman in an underdeveloped nation is predicted to be anemic, which has negative consequences such as an increased risk of maternal death, perinatal mortality, and low birth weight. Nutritional iron deficiency occurs when dietary iron absorption is insufficient to meet physiological needs. Iron deficiency anemia (IDA) can arise if iron intake is restricted or inadequate owing to low dietary consumption or the presence of some chronic conditions.

Iron supplements, which are commercially accessible, are used to treat iron deficiency anemia. However, they have side effects affecting the gut lumen and mucosal area of the intestine because of the free iron-dependent radical production. Thus, it might be important to reduce free iron interaction with the gastrointestinal (GI) cells during digestion and absorption. In the end, this will lower free iron toxicity while increasing iron solubility and bioavailability. Because mammals lack active iron excretory systems, intestine dietary iron absorption regulates iron homeostasis. Thus, the enhancement of intestinal iron absorption during IDA is critical to increase the iron level in blood and peripheral tissues (Evcan and Gulec, 2020).

Food fortification with iron, which is the addition of iron to processed foods, is the most practical, cost-effective, long-term, and food-based approach in order to overcome IDA. The World Health Organization (WHO) has also been recommended it as a significant method for preventing micronutrient deficiencies (FAO/WHO, 2007). However, it has some limitations related to iron such as low solubility, oxidative reactions such as lipid oxidation, modification of the flavor and color of the iron-fortified food. Low dietary iron bioavailability is the most effective factor that causes IDA among these limitations. (Caetano-Silva *et al*., 2018). Therefore, new alternative ways have been searched for fortification with iron that can supply food with low physical and sensorial change with high bioavailability.

Peptides derived from food protein extracts or hydrolysates have increasingly attracted interest as novel metal chelators. The chelated mineral is more stable and less likely to react chemically with the environment (Caetano-Silva *et al*., 2018), with a similar or better effect compared to inorganic salts alone (Eckert *et al*., 2016). Also, amino acids and certain other organic acids, increase iron absorption by buffering the pH of the intestinal contents. (Torres-Fuentes *et al*., 2012). The idea of using iron-protein or iron-peptide complexes could be an alternative strategy to overcome the problems related to iron fortification.

Food fortificants including many iron sources such as ferrous sulphate and ferrous carbonate can be used for designing functional food production. However, they have poor bioavailability. This might be due to a variety of factors. Mineral solubility is reduced by the moderate alkaline environment of the intestine. Low solubility indicates that a low rate of absorption and low bioavailability (Eckert *et al.*, 2016). Furthermore, iron bioavailability is limited at physiological pH in the presence of peptic digestion because ferrous ions (Fe^{2+}) are rapidly oxidized to the insoluble ferric (Fe^{3+}) form, which must be first reduced by the enzyme before being absorbed by enteric cells (Torres-Fuentes et al., 2011, Caetano-Silva et al., 2015 and Caetano-Silva et al., 2018). As a result, substances that keep iron soluble and stable in the gastrointestinal tract by keeping it in the ferrous state boost iron bioavailability.

Gastrointestinal digestion has a significant impact on the biological activity of food-derived peptides, allowing the generation of new active fragments with increased function or on the other hand, resulting in fragments with reduced or no activity. (Gonzales-Montoya *et al*., 2018). These bioactive peptides can also be generated by previous *in vitro* protein hydrolysis (Torres-Fuentes *et al*., 2011). The existence of digestive enzymes that break down proteins into smaller peptides and individual amino acids, which move into the systemic circulation via transcellular or paracellular pathways, limits the absorption of intact food proteins (Markell *et al.*, 2017). So, legume peptide-iron complexes might be used as dietary supplements to increase mineral solubility and bioavailability. Furthermore, because these peptides have strong antioxidant properties, they may help to reduce iron oxidation in the gastrointestinal tract and/or during food storage. The peptides must reach their targets intact in order to exhibit their bioactivity, therefore biostability and bioavailability are critical for delivering physiological advantages (Mohan *et al.*, 2015).

Within this context, the main objectives of this Ph.D. thesis were *(i)* to develop highly bioavailable, edible protein-iron complex hydrolysates from legumes that can be used as an additive in food products in order to reduce iron deficiency risk in humans and *(ii)* to investigate their functional properties against iron deficiency anemia. Legume proteins extracts are widely used as functional ingredients however their functionality must be demonstrated by testing their physiological function in the cell culture system. Therefore, it is important to be aware of knowing how the mechanism works. *(iii)* Investigating the compensatory effects of copper mineral on iron deficiency anemia and *(iv)* its main application for functional food development were other driving forces for the experiments.

CHAPTER 2

LITERATURE REVIEW

Iron deficiency is one of the major global nutritional deficiencies especially in women, infants and children, and defines as a condition in which there are no mobilizable iron stores and in which signs of a compromised supply of iron to tissues (WHO, 2001). Due to undesirable effects such as an increased risk of maternal death, perinatal mortality, and low birth weight, iron deficiency is a major health problem during pregnancy. A nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from the diet. If iron intake is limited or inadequate due to poor dietary intake, or in the presence of some chronic diseases iron deficiency anemia may occur.

2.1. Main Functions of Iron

Iron is the fourth most prevalent transition metal by weight, accounting for the majority of the Earth's crust, which is a physiologically important trace element for all living things (Abbaspour et al. 2014; Verma et al, 2017). Because iron is the major component of hemoglobin, it is required to transport oxygen to tissues in the human body. It also acts as an active center of proteins, facilitating oxygen and electron transfer in metalloenzymes, and take important roles in cell division and differentiation, generation of mitochondrial energy, mechanisms of DNA replication and repair, protection from reactive oxygen species (ROS), and immune response against to pathogenic microorganisms (Gulec et al., 2013). Iron is essential for myelination of the spinal cord and white matter of the central nervous system in the brain; hence it plays an important role in neuron signaling (Saini et al., 2016). As a consequence, iron is a vital component for the survival of living things.

Despite the fact that iron is involved in a variety of noteworthy functions in biological systems, it is insoluble at physiological pH resulting in low bioavailability.

Thus, iron transport mechanisms and storage proteins have developed in all living organisms (Durukan et al., 2011). Because mammals lack a unique active excretory system for iron, its level is regulated by intestinal absorption in the human body. Therefore, it could be said that in the context of molecular and genetic regulation, intestinal iron metabolism is critical. Insufficient dietary iron absorption from the intestine results in IDA, the world's most common nutritional deficiency. Excess iron in the body (hemochromatosis), on the other hand, can lead to serious complications such as type 1 diabetes, thalassemia, liver dysfunction, myelodysplastic syndromes, and sickle cell disease (Guilbert, 2003).

2.1.1. Iron Metabolism and Intestinal Absorption

The reticuloendothelial system (RES) regulates the iron balance in the human body. Iron is used by the bone marrow to produce hemoglobin. After hemoglobin is produced, spleen macrophages break it down. Free iron is transported into the bloodstream and stored in the liver or utilized in other tissues for physiological purposes (Figure 2.1).

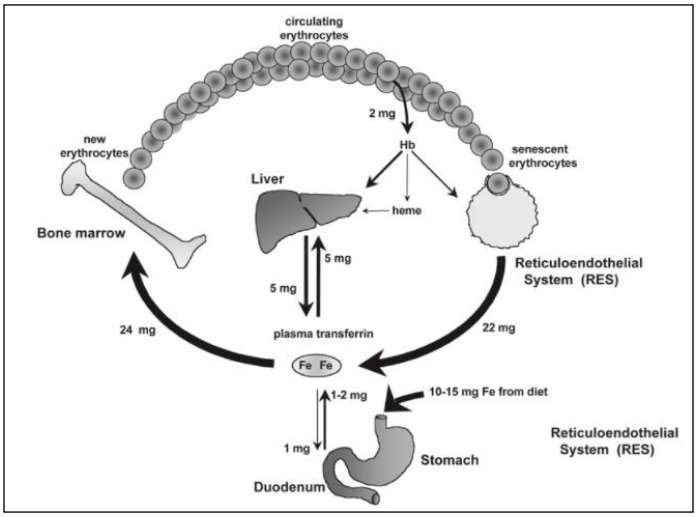


Figure 2.1. Iron recycling system in the human body.
(Source: Knutson and Wessling-Resnick, 2003)

Because there is no specific active iron excretory system, intestinal iron absorption is the most important stage in regulating whole-body iron levels. As indicated

previously inadequate absorption causes anemia whereas excess iron causes tissues toxicity. Intestinal absorption of inorganic iron from dietary sources is thought to be mediated by two processes, as indicated in Figure 2.2 (Gulec et al., 2014). In both of the hypothesized pathways (A and B) the reductase protein (duodenal cytochrome B (Dcytb/Cybrd1)) found in the apical side of the intestine, which is the side the enterocyte cells facing the nutrients, converts ferric form (Fe^{3+}) to ferrous form (Fe^{2+}) (McKie et al. 2001). Because only Fe^{2+} is physiologically active in human cells, this conversion step is essential for iron absorption.

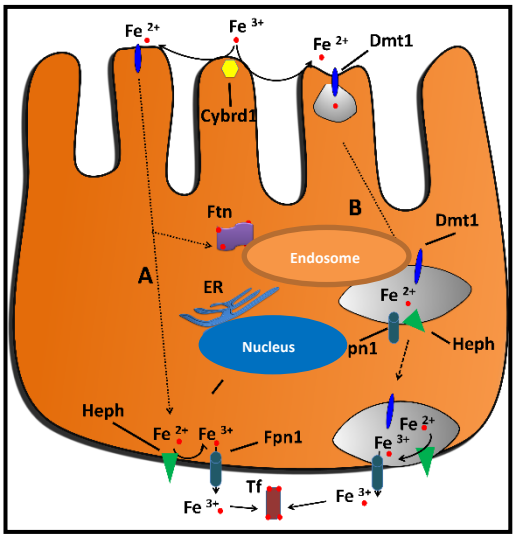


Figure 2.2. Iron absorption mechanisms of small intestine enterocyte cells (A and B). (Source: Donovan et al., 2000)

The best-described mechanism for iron absorption is depicted in Figure 2.2 as "A". Divalent metal transporter (Dmt1), which is a member of the solute carrier group of membrane transport protein found on the apical side of the enterocyte cells, reduces iron to Fe^{2+} and then enters the cell. Iron may enter cells in two ways. In a first way, iron is stored in ferritin (Ftn) and second, iron is transferred into the bloodstream (Harrison and Arosio, 1996). The enterocyte's basolateral side that interacts with blood vessels is where iron efflux occurs. Hephaestin (Heph), which is an oxidase protein, oxidizes Fe^{2+} to Fe^{3+} and then an oxidized form of iron is transferred to the exterior of the cell by Ferroportin (Fpn1) (Donovan et al., 2000). Iron binds to transferrin (Tf) in the blood and is transported

to other tissues such as bone marrow, liver (Anderson et al., 2002). As a second way shown in Figure 2.2 as “B”, the transport protein Dmt1 found on the apical side of the cell takes up Fe^{2+} by the endosomal complex generated within the cell (Ma et al. 2002). After that, Fe^{2+} is converted to Fe^{3+} by the oxidase protein Heph, and Fe^{3+} on the endosome is transported to the blood by the transport protein Fpn1. It is then carried to the body’s tissues via the transporter protein Transferrin (Tf).

2.1.2. Genetic Regulation of Intestinal Iron Homeostasis

Dietary iron deficiency is a major signal for gene regulation in enterocyte iron metabolism. The coordinated controlled expression of the transferrin receptor and ferritin, which mediate iron intake and storage, respectively, achieves cellular iron homeostasis. IRP1 and IRP2, two cytoplasmic iron regulatory proteins, are involved in the posttranscriptional process. During iron deficiency, IRPs stabilize the transferrin receptor and bind to iron responsive elements (IREs) of ferritin mRNAs within their untranslated regions which limits the translation. The IRE/IRP system also regulates the expression of other IRE-containing mRNAs that encode for proteins of iron and energy metabolism. IRP1 and IRP2 activities are controlled by different posttranslational mechanisms in response to iron levels in the cells (Panthopoulos, 2004).

Nonpeptide-encoding motifs at the 5’end of Fpn1 and Ftn mRNAs have a function in the regulation. These motifs at the 3’ends of mRNAs of Tfr1, which is responsible for intracellular iron absorption in liver cells, and Dmt1, which is responsible for transporting iron in the intestine. IRPs bind to the IRE base sequence at the 5’end of the mRNAs when iron levels decrease, affecting the binding of mRNA to the ribosome complex and reducing protein synthesis. It binds to the 3’end of the RNAs and enhances mRNA stability by decreasing exonuclease-mediated mRNA degradation (Panthopoulos, 2004). Based on this mechanism, when the level of intracellular iron is raised Ftn and Fpn1 protein generation occurs, the mRNA stability of Dmt1 and Tfr1 is reduced concurrently. Conversely, mRNA stability of Dmt1 and Tfr1 enhances at the point that Ftn and Fpn1 protein synthesis reduces when the level of intracellular iron reduces (Muckenthaker et al., 2008).

2.2. Effects of Copper on Physiological Iron Metabolism

Previous investigations have noted that copper influences iron metabolisms (Fox, 2003; Klevay, 1997; Chase et al., 1952). During iron deficiency, in many mammalian species, body copper levels are increased, including in the intestinal mucosa, the liver, and in serum. Copper may be a key player in the compensatory response of the intestinal epithelium to increase body iron acquisition during states of deficiency.

The physiological link between copper and iron metabolisms is well defined in two different tissues. Hephaestin (Heph), which is responsible for the oxidation of iron ($Fe^{2+} \rightarrow Fe^{3+}$) in the intestinal enterocyte cells, cannot be functional without having copper in its structure. Reeves and Demars (2004) discovered that nutritional copper deficiency inhibited the release of iron from enterocyte cells into the blood, resulting in iron deficiency in rats. Another study revealed that, aside from hephaestin, enterocyte cells may contain unidentified copper-dependent proteins that are involved in iron oxidation (Gulec and Collins, 2014). Ceruloplasmin, a copper-dependent protein present in the liver, performs the same function as hephaestin is essential for the release of iron deposited in the liver (Harris et al., 1995). The amount of copper in the enterocyte cells and blood was shown to be enhanced in the case of anemia caused by insufficient iron administration (Ravia et al., 2005). However, the physiological reason for the increase in enterocyte cells is unknown. In the same study, it was declared that there was a significant increase in the level of gene expression of the transport protein Atp7a in the rat enterocyte cells induced with deficiency dependent on diet. Another research focused on iron metabolism in an *in vitro* rat enterocyte cell model formed by deleting the Atp7a protein's mRNA. The expression levels and regulation of genes involved in iron metabolism were found to be changed (Gulec and Collins, 2014). However, the molecular mechanism behind this impact is unknown. In a hypoxic situation produced by iron deficiency, certain genes involved in iron and copper metabolisms in enterocyte cells showed comparable coordination (Xie and Collins, 2011). Thus, iron deficiency anemia impacts iron and copper-dependent pathways in enterocyte cells, and copper mineral may play a role in iron-deficiency-dependent regulation.

2.2.1. Main Functions of Copper

Copper is a cofactor in the structure of several key enzymes for physical functioning and it is necessary for their functionality. For instance, it regulates the activity of enzymes involved in the synthesis of collagen and elastin proteins in connective tissue (Collins and Klevay, 2011). Copper has been demonstrated to be beneficial in carrying out heart cells functions, and copper deficiency has been linked to heart function problems (Klevay, 2000). Copper is also required for the proper functioning of important enzymes present in brain cells (Krebs and Krawetz, 1993). Copper deficiency impairs the activity of these enzymes, resulting in unfavorable health outcomes in a variety of physiological conditions.

Copper is absorbed by the body at a rate of 0.6 to 1.6 mg per day by intestine enterocyte cells. Copper level in the human body is estimated to be around 4.5 mg/kg of body weight (Prohaska and Gybina, 2004). Although the copper deficiency is less common than iron deficiency, it can lead to death if not treated earlier. Copper deficiency is frequently inherited, and Menkes disease develops as a result of the intestine's inability to absorb copper. (Tumer and Moller, 2010). Wilson's Disease has undesirable consequences, for example, is caused by an excess of copper mineral in the body (Sternlieb, 2000). Therefore, as with iron, the amount of copper in the cell should be closely regulated.

2.2.2. Copper Metabolism

Proteins involved in copper absorption from intestinal enterocyte cells and cellular copper metabolism are depicted in Figure 2.3. The most common type of copper present in foods is the cupric state (Cu^{2+}). The reductase protein found in enterocyte cells converts to Cu^{2+} form to Cu^{1+} state (Wyman et al., 2008). Intracellular copper transport protein, Ctr1, located on the apical side where first encountered part with the food of enterocyte cells, transports Cu^{1+} into the cell by forming endosomal complex (Sharp, 2003). Because free copper in the cell is toxic, it is transferred to the target proteins, immediately. Superoxidase dismutase, Sod1, transports copper mineral via copper transporter protein, Ccs (Kim et al., 2008). Another transport protein, antioxidant protein-1 (Atox-1), transports copper to Atp7a protein which is responsible for transporting copper to Golgi

apparatus for the generation of copper-dependent proteins (Prohaska and Gybina, 2004). The cytolosic metallothionein protein, Mt1a, stores copper mineral in the cell or copper is given to bloodstream through enterocyte cells with Atp7a transport protein (Kim and Petris et al., 2007). The copper is converted to Cu^{2+} oxidizing with oxygen when flows into the blood and is delivered to all of the body's organs via albumin or macroglobulin proteins (Prohaska, 2008).

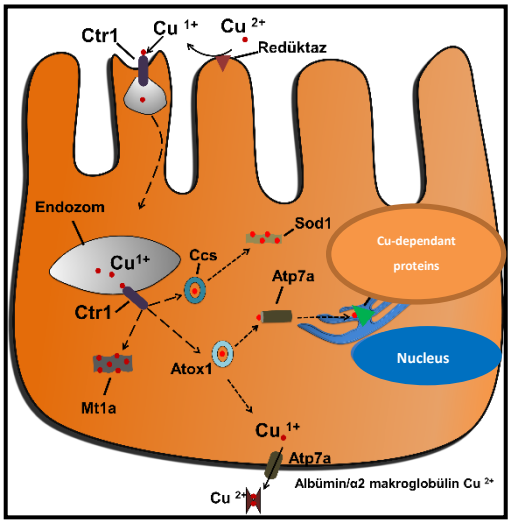


Figure 2.3. Copper absorption mechanisms of small intestine enterocyte cells.

2.3. Iron in Food

Iron may bind to heme proteins in flesh foods like fish, meat, and poultry as well as non-heme (or inorganic) forms present in plant foods, like cereals, spices, beans, herbs, nuts, fruits, and vegetables (Saini et al. 2016). Although the processes for absorbing these two types of iron are distinct, they both collect in the same iron pool in the body and contribute to iron metabolism in the same way. Heme iron absorption is efficient and relatively unaffected by other nutrients in the diet. Non-heme iron (mostly ferric state), on the other hand, is very insoluble and its bioavailability is influenced by other dietary factors. Gastric acid and ascorbic acid help to convert to ferrous state and solubilize dietary ferric iron, allowing for better absorption. Additionally, dietary factors present in plants, such as polyphenols, phytate, tannins, and oxalate, reduce nonheme iron absorption. Proton

pump inhibitors, which are used to treat stomach acid reflux, *Helicobacter pylori* infection, and inflammatory disorders such as celiac disease, reduce nonheme iron absorption (Gulec et al. 2014). The main subject of this thesis is nonheme absorption.

2.4. Iron Bioavailability

The proportion of food that can be absorbed through/by the intestine and rendered accessible for metabolic use or storage is referred to as bioavailability (Lowe and Wiseman, 1998). The bioavailability of an essential metal is governed by its metabolic utilization in nutrition sciences. For this purpose, the term "total utilization" is used here to refer to the portion of a nutrient that is utilized in metabolism following digestion, absorption, and distribution. (Schümann and Elsenhans, 2002). The term "bioavailability" refers to the availability for absorption, also known as "bioaccessibility"; as well as absorption; tissue distribution; and bioactivity. (Stahl et al., 2002).

Dietary iron occurs in two forms: heme and nonheme as mentioned in Section 2.2. Heme iron is highly bioavailable (15%-35%) and dietary factors have little effect on its absorption, on the other hand, nonheme iron absorption is much lower (2%-20%) and the presence of other food components strongly affect its absorption. Moreover, in most meals, the amount of nonheme iron consumed is several times larger than that the amount of heme iron consumed. Nonheme iron thus provides more to iron nutrition than heme-iron, despite its reduced bioavailability. Iron absorption is inhibited by phytic acid, polyphenols, calcium, but ascorbic acid and muscle tissue promote absorption converting ferric iron to ferrous iron and binding it in soluble complexes (Abbaspour et al, 2014).

Parameters such as solubility and binding constants of produced iron complexes, play a crucial role in iron bioavailability since they determine the degree to which iron is absorbed. Most iron absorption is assumed to occur in the duodenum (pH 6–6.5) and upper jejunum (pH between 7 and 9) after reduction of Fe^{3+} to Fe^{2+} by an enzyme (ferro-reductase) found in the gut wall. If simply considering pH, the high solubility of iron in a medium at pH > 5 would result in good bioavailability, (Tian et al., 2016).

Iron deficiency is caused by absorbing less iron from the food than the body required for good health. Low iron intake or consumption of foods with poor iron absorption, i.e. foods that have low iron bioavailability, can lead to this scenario. Iron bioavailability can vary from 2 to 35% depending on the state of iron in the food and the

presence of enhancers or inhibitors of iron absorption. As a result, it is critical to assess foods for their ability to offer bioavailable iron in order to supply consumers with accurate information on a good iron-rich diet (Lai et al., 2012). Ferritin is the major intracellular iron-binding protein that stores iron in an insoluble form, as previously stated. The majority of iron is bound to ferritin, which is widespread and well preserved (Abbaspour et al, 2014; Gulec et al. 2014). Ferritin has several implications for iron from a nutritional point of view. To begin with, it has a gradual iron release profile, which implies it may protect intestinal cells from oxidative damage when compared to some other conventional supplements. Second, because ferritin internalization differs from that of small iron salts, chelates, or heme, supplementation may be a more effective strategy to treat iron deficiency anemia than traditional iron therapies.

The seeds of legumes are rich in high-quality protein for human and animal nutrition ranging from around 20% (dry weight) in peas and beans to 38–40% in soybeans and lupins. Beans, soybeans, lentils, peas, and chickpeas are all legumes and are common staple foods. Albumins, globulins, glutelins, and prolamins are the traditional classifications for storage proteins based on their solubilities. Albumins are water-soluble, globulins are insoluble in pure water but dissolve in dilute saltwater solutions, glutelins are soluble in dilute acids or bases, and prolamins are soluble in ethanol/water solutions. Globulins, which are generally classified as 7S and 11S globulins according to their sedimentation coefficients (S), are the most common type of storage protein in grain legumes. However, the amino acid composition only shows the potential quality of a protein, with bioavailability being crucial for amino acids supply in the diet. Food proteins and their peptide fragments can be effectively used as biological carriers for metals (Duranti M., 2006).

2.5. Importance of Fortification for Iron Deficiency

There are too many ways for preventing iron deficiency such as food diversification, supplementation, fortification, and biofortification. Food fortification is usually regarded as the deliberate addition of one or more micronutrients to particular foods, so as to increase the intake of these micronutrient(s) in order to correct or prevent a demonstrated deficiency and provide a health benefit and among multiple strategies to control iron deficiency.

Food fortification is an effective measure to increase the intake of iron without causing a change in the existing dietary patterns. However, fortification of foods with iron is more difficult compared to other nutrients, such as zinc in flour, iodine in salt, and vitamin A. It has some limitations (Gupta et al, 2015). For example, designing of stable products containing highly bioavailable iron sources remains a challenging goal. On one hand, water-soluble mineral salts or complexes with high bioavailability may cause physico-chemical instabilities and often react with other food components causing sensorial problems with the products such as off-flavors, color changes, or fat oxidation. On the other hand, the slow dissolution of mostly insoluble mineral compounds in the form of large particles becomes the rate-limiting step and leads to the low bioavailability (Tian et al., 2016; Abbaspour et al, 2014).

Fortification is usually made with much lower iron doses than supplementation. Ferrous sulfate, ferrous fumarate, ferric pyrophosphate, and electrolytic iron powder are among the iron compounds indicated for food fortification. Wheat flour is the most frequent iron-fortified food and it is generally fortified with elemental iron powders, which WHO does not suggest. Hurrell and Egli (2010) reported that of the 78 national wheat flour programs only eight would be expected to improve iron status. Commercial infant foods, such as formulas and cereals, are also commonly fortified with iron (Abbaspour et al, 2014).

Because IDA impacting over 2 billion people worldwide is the most prevalent and pervasive nutritional condition. Iron fortification, such as ferrous iron salts (ferrous sulfate and ferrous gluconate), is now the most widely used method to combat IDA. However, such treatment has been associated with adverse effects such as constipation, diarrhea, and decreased growth. Therefore, the non-heme iron from plant resources also iron binding capacities are taken into account, is a good choice. Non-heme forms, such as iron carbonyl, iron-dextran, and ferritin existed in plants, could play an important role in iron fortification. (Yang et al., 2015). Wherein phytoferritin represents a novel and natural strategy for iron fortification (Lönnerdal, 2009, Theil et al., 2008), and has received increasing attention for many years. For example, 90% of the total iron is stored in ferritin in plant species, particularly in legume seeds (Liao et al., 2014).

In the study conducted by Zhu, Glahn, Nelson and, Miller (2009) studied the effect of pH on the bioavailability of iron in soluble ferric pyrophosphate (a soluble complex of ferric pyrophosphate and citric acid) and other iron fortificants, using a Caco-2 cell culture model with, or without, the combination of *in vitro* digestion. They discovered

that exposing $FeSO_4$ to pH 2, and then adjusting to pH 7 significantly reduced $FeSO_4$ bioavailability. The effects of pH on iron bioavailability have been found to be minimized by chelating iron ions (Tian et al., 2016).

CHAPTER 3

COMPENSATORY RESPONSE OF COPPER MINERAL ON INTESTINAL IRON DEFICIENCY ANEMIA

Iron is an essential and vital nutrient for life, as it enables the systems of living organisms to function properly. Because iron is the major component of hemoglobin, it is required to transport oxygen to tissues in the human body. Moreover, it is necessary for controlling cell division and differentiation, mitochondrial energy generation, DNA replication and repair, and immunological response to pathogenic microorganisms (Gulec and Collins, 2014, Verma et al., 2016). Because mammals lack a specialized active iron excretory system, iron levels in the human body are strictly regulated through intestinal absorption. Therefore, intestinal enterocyte cells keep the body's iron levels under control. Iron deficiency anemia is caused by insufficient dietary iron absorption through enterocyte cells of the intestine, which is one of the most common nutritional deficiencies worldwide, especially in women, newborns, and children. Many mammalian species, including humans (Fox, 2003), have higher copper mineral levels in their intestinal mucosa (El-Shobaki and Rummel, 1979), liver, and serum (Ece et al., 1997) as a result of iron insufficiency. It has been observed in previous studies that copper homeostasis disturbances have been linked to iron metabolism (Gulec and Collins, 2014, Fox, 2003, Collins et al, 2010, Ha et al, 2017, Wang et al., 2018).

Divalent metal transporter (*DMT1*), the iron exporter ferroportin 1 (*FPN*1), might represent a potential connection between iron and copper metabolisms since there are studies that suggest the iron amount is regulated by copper in the intestine (Gulec and Collins, 2013, Matak et al, 2013, Wang et al., 2018). Hypoxia-inducible factor 2α (HIF-2α) in the intestine, because it modulates apical and basolateral iron transporters, is critical for iron absorption during iron deficiency (Schwartz et al, 2019). Copper affects the DNA-binding activity of HIF-2α, demonstrating once again another link between the copper mineral and iron homeostasis. *DMT1* and *FPN* were shown as direct target genes

related to HIF-2α (Anderson et al., 2013). mRNA regulation of Ankyrin repeat domain 37 (*ANKRD37*), prolyl 4-hydroxylase (*P4ha1*), and HIF prolyl hydroxylase 3 (*EGLN3*) are the most well-known marker genes for the hypoxic signal under iron deficiency.

In brindled mice, the regulation of genes involved in iron and copper homeostasis was disrupted, according to a recent study conducted by Gulec and Collins (2013). However, when intracellular copper level increased during iron deficiency anemia (IDA), copper did not affect the genes' regulation, which plays a role in enterocyte iron metabolism. Enterocyte cells from the mice intestine were treated with high intracellular and low blood copper levels in the same research. Thus, blood copper level would be a more important regulator of iron metabolism-related genes during IDA. The apical side of the enterocyte is in contact with dietary nutrients, whereas the basolateral part is in contact with the blood. Considering this apical and basolateral polarization, different sides of enterocytes which in relation to different environmental conditions (diet or blood) may be necessary for controlling the molecular and genetic regulation of iron metabolism in enterocyte cells. However, to the best of our knowledge, no research exists related to the dependency of the apical versus basolateral copper for enterocyte cell iron metabolism during IDA. Given this background, the studies of this chapter investigated the effects of dietary and blood copper treatments separately on iron and iron-dependent hypoxic gene regulations of anemic enterocyte cells by *in vitro* modeling of the human intestine system.

3.1. Cell Culture

The human colorectal adenocarcinoma epithelial cell line, Caco-2, was purchased from the American Type Culture Collection (ATCC, HTB-37, Manassas, VA). Caco-2 cells were cultured in minimal essential medium (MEM) (Sigma, United Kingdom) supplemented with 20% fetal bovine serum (FBS) (Gibco, Cat. No. 10500), 1% penicillin and streptomycin (100 U/mL) and, 1% nonessential amino acid solution (Gibco, Cat. No. 11140). They were maintained in 75 cm^2 culture flasks at 37°C in a constant humidified incubator with an air atmosphere of 5% CO_2/95% O_2. When cultures reached 70-80% confluency, they were plated for either subsequent passage or treatment. Caco-2 cells used for the treatment experiments were between the 20^{th}-30^{th} passages.

3.2. Modeling of the Human Intestinal System and Copper Treatments of Apical and Basolateral Sides of the Cells During Iron Deficiency Anemia

To test the effect of the dietary and blood copper on iron deficiency anemia, the human intestinal system was mimicked. The Caco-2 cells were seeded into a bicameral collagen-coated polytetrafluoroethylene membrane with 0.4-μm pore size and 1.12 cm^2 diameter (12-well inserts) (Corning, Cat. No.: 3493). After three days of confluent culture, the cells were grown for an additional 21 days to form a monolayer and became polarized under standard conditions in the indicated MEM media. The experimental cell medium was changed every other day. Transepithelial electrical barrier resistance (TEER) was measured by an epithelial volt-ohm meter (EVOM; World Precision Instruments, Inc., FL, USA) to confirm the cell tight junction integrity. Cell monolayers with the TEER values above 250 Ω/cm^2 were only used for the treatment experiments (Sambuy et al. 2005). Iron deficiency anemia was induced in the cells at 21-day of post-seeding using a chemical agent (Deferoxamine, DFO) at a concentration of 200 μM. After incubation for 24 h, the apical and/or basolateral compartments of anemic Caco-2 cells were treated with the copper (copper (II) chloride, $CuCl_2$; 100 μM) or iron (ferric ammonium citrate, FAC; 100 μg/mL) and/or together at the same time for further 18h in the incubator (Figure 3.1).

3.3. Determination of the Effect of Copper on Iron Metabolism at the Molecular and Genetic Level

The Caco-2 cells were seeded at 1×10^5 cells/well into classical cell culture plates (12-well plates) (Costar, Cambridge, MA) to investigate the effects of Cu on molecular and genetic regulation of iron metabolism. Iron deficiency anemia was induced in the cells at 21-day of post-seeding 200 μM DFO. After incubation for 24 h, anemic cells were treated with the copper and/or iron minerals at the same concentrations as previously described in Section 3.2 (Figure 3.2).

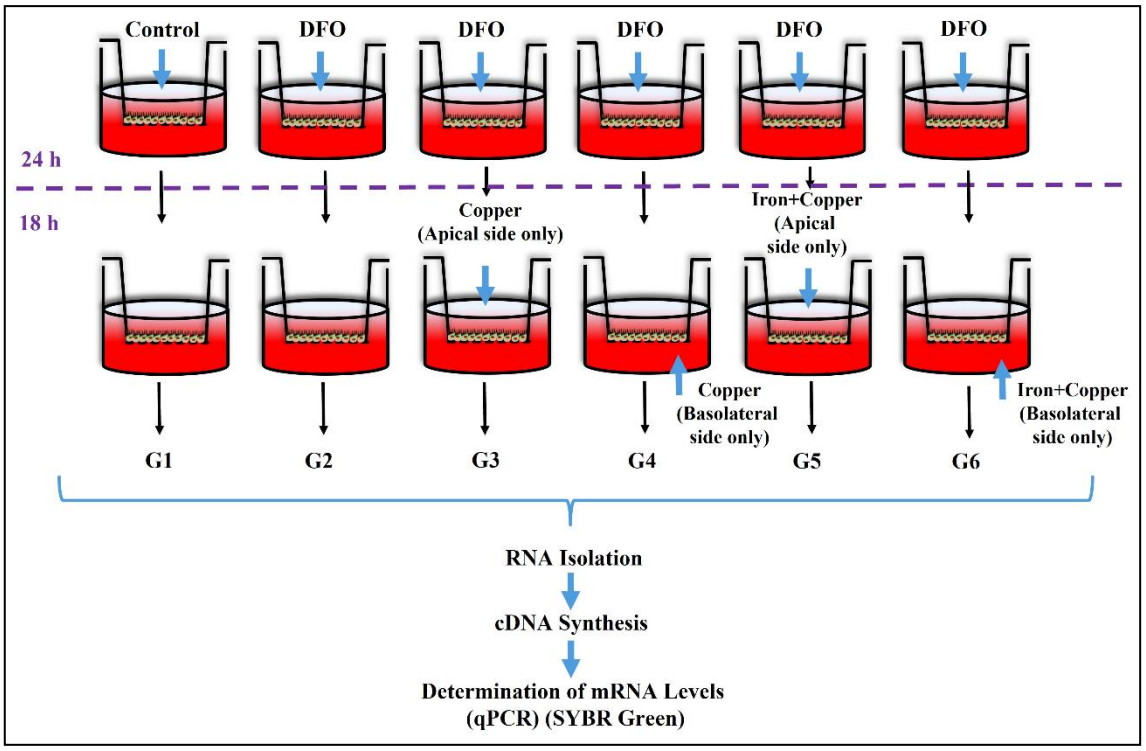


Figure 3.1. Determination of the effect of copper mineral on the molecular and genetic regulation of iron metabolism in anemic cells growing on 12-well inserts. G1: Control group, G2: Anemic group, G3: Copper treated group in anemic condition from the apical side, G4: Copper treated group in anemic condition from the basolateral side, G5: Iron and copper treated group in anemic condition from the apical side, G6: Iron and copper treated group in anemic condition from the basolateral side.

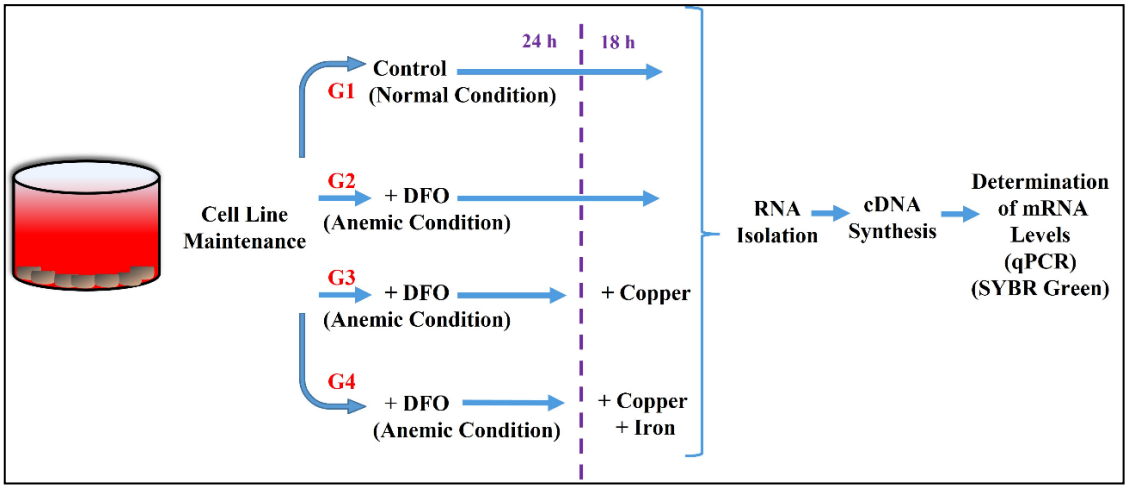


Figure 3.2. Design of the experiments for determination of the effect of copper on iron metabolism at the molecular and genetic level in anemic cells growing on 12-well plates. G1: Control group, G2: Anemic group, G3: Copper treated group in anemic condition, G4: Copper and iron treated group in anemic condition.

3.4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

mRNA expression levels of the marker genes that regulate iron metabolism and hypoxic condition were evaluated. Total RNA was isolated from cells with RNAzol reagent (MRC, Cat. No.: RN190) following the manufacturer's protocol. One microgram of each total RNA was converted to cDNA using the cDNA synthesis kit (Lifetech, Cat. No.: 4368814). qRT-PCR was performed on an ABI StepOnePlus instrument (Lifetech, CA, USA) by using gene-specific oligonucleotide primers and SYBR-Green mix (Lifetech, Cat. No.: 4367659). C_T (threshold cycle) levels were normalized according to *CYPA* mRNA expression as a housekeeping gene (Table 3.1.). Mean fold changes in gene-specific mRNA levels from all experimental groups were calculated by the $^{2\text{-}\Delta\Delta Ct}$ analysis method (Hu et al., 2010).

Table 3.1. Marker genes that were used in the evaluation of the effect of copper treatments on iron metabolism.

Iron Metabolism	Hypoxia	Copper Metabolism
DMT1	*ANKRD37*	*MT1A*
FPN	*EGLN3*	
HEPH		
TFR		

3.5. Statistical Analyses

The results were expressed as the mean values ± standard deviation of three independent experiments with at least two parallels of each experimental group. Statistical analysis for more than two groups was carried out by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test by using GraphPad Prism 6 (GraphPad Software Inc., CA, USA). Data were considered significant for $p \leq 0.05$.

3.6. Results and Discussions

3.6.1. Modeling of Human Intestine System and TEER Measurements of Monolayer Caco-2 Cells

The apical and basolateral polarization of Caco-2 cells can behave like the human small intestine (Sambuy et al., 2005). Therefore, Caco-2 cells were grown on the bicameral cell culture system for 21 days, and then DFO and mineral treatments were performed. TEER was measured from all experimental groups at the end of 21 days and after treatments to control the stability of the monolayer integrity of the cells. As shown in Figure 3.3, no significant changes were observed in TEER values between the experimental groups before and after Cu and Fe treatments. Furthermore, TEER values that are higher than 250 ohm/cm^2 reflected the experimentally polarized cells (as apical and basolateral) in the human intestinal system.

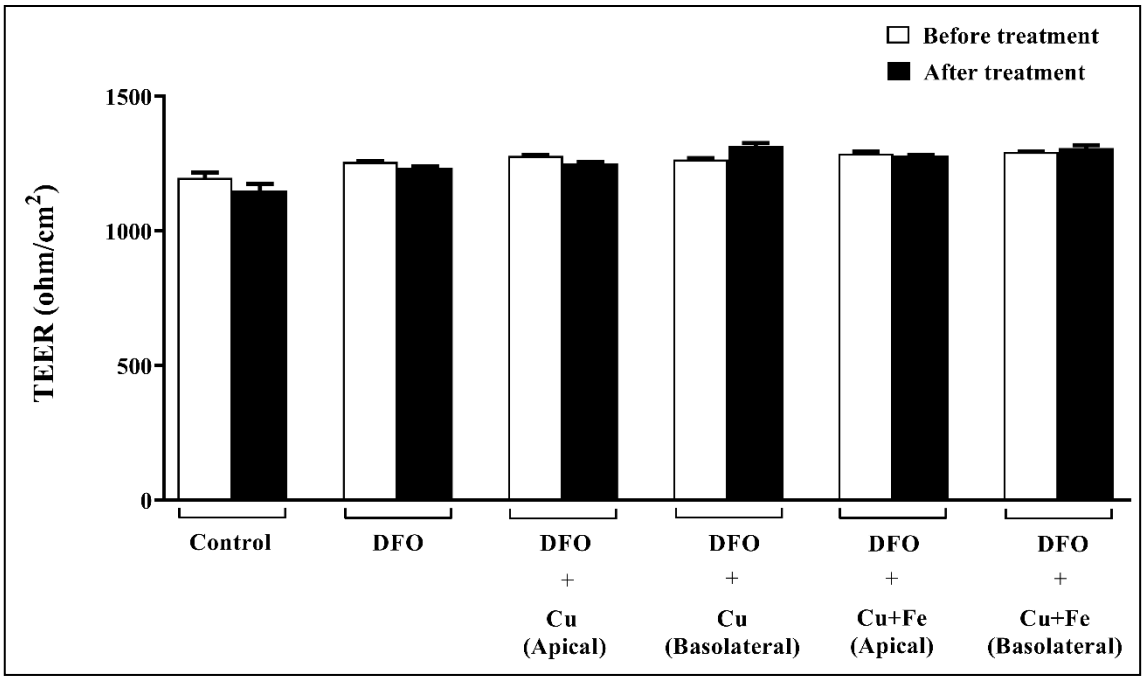


Figure 3.3. Transepithelial electrical resistance (TEER) measurements in the Caco-2 cells grown on bicameral insert for 21 days.

3.6.2. Apical and Basolateral Treatments of Copper in DFO Induced Anemic Monolayer Caco-2 Cells

When the Caco-2 cells are grown on the bicameral cell culture system, it allows us to study the effects of apical and basolateral Cu treatment on the regulation of iron metabolism-related genes in enterocyte cells during DFO induced IDA. DFO treatment induced *DMT1*, *TFR*, and *ANKRD37,* and *EGLN3* mRNA levels; however, *FPN, HEPH,* and *MT1a* were not affected (Figure 3.4). When Cu was given to the apical side of the cells, it did not affect mRNA levels of genes, including *DMT1*, *TFR*, *ANKRD37*, and *EGLN3* that were induced by DFO. Cu treatment of the basolateral side of cells significantly reduced *DMT1*, *FPN* and *TFR*, and *ANKRD37* mRNA levels under the IDA condition. Moreover, *DMT1*, *FPN, HEPH,* and *TFR* gene expressions were not changed by apical side treatments of the cells by Cu and Fe, whereas *ANKRD37* and *EGLN3* mRNA levels were slightly reduced. Furthermore, when both Cu and Fe were given to the basolateral side of the cells, mRNA levels of iron regulating genes (*DMT1*, *FPN*, and *TFR*), and hypoxia-related genes (*ANKRD37*, and *EGLN3)* were significantly lower than all treatment groups. *MT1a* mRNA levels were significantly increased by basolateral Cu treatment of cells, and this induction was higher when both Cu and Fe were given to the basolateral side of the cells (Figure 3.4a and b).

3.6.3. Effect of Copper on Iron Deficiency Anemia in Caco-2 Cells Grown on the 12-well Tissue Culture Plates

Copper and iron interaction in enterocyte cells has attracted interest since their homeostasis is controlled by intestinal absorption. In this study, Caco-2 cells were grown on 12-well tissue culture plates for 21 days and then treated by DFO to induce IDA. The cells were treated to Cu with and without Fe. Then mRNA levels of iron regulating and iron-dependent hypoxia regulating genes were evaluated to see the effects of Cu on iron metabolism during IDA (Figure 3.5). DFO treatment significantly induced *DMT1*, *TFR*, and *ANKRD37,* and *EGLN3* mRNA expression levels, whereas *FPN, HEPH,* and *MT1a* mRNA levels were not affected. The Cu treatment did not reduce DFO induced gene mRNA expressions and did not affect *FPN* and *MT1a* mRNA levels. However, Cu with Fe treatment significantly reduced *DMT1*, *TFR*, *HEPH*, *ANKRD37*, and *EGLN3* mRNA

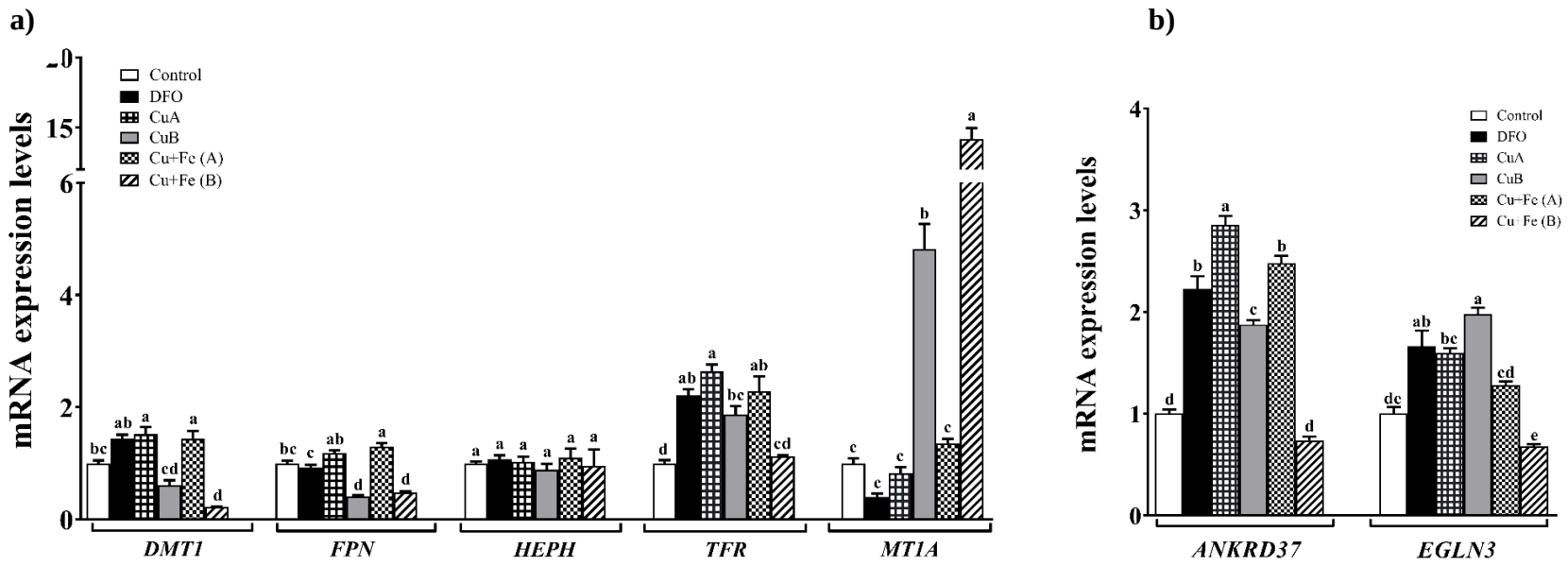


Figure 3.4. The effect of copper mineral on iron and hypoxia-regulated gene mRNA expression levels under the anemic conditions in polarized Caco-2 cells grown on the bicameral cell culture system for 21 days. The effects of Cu and Cu with Fe on iron (a) and hypoxia (b) regulating gene mRNA expressiones. Data presented as mean ± SD (n = 3). Different letters on bars indicated significance ($p < 0.05$).

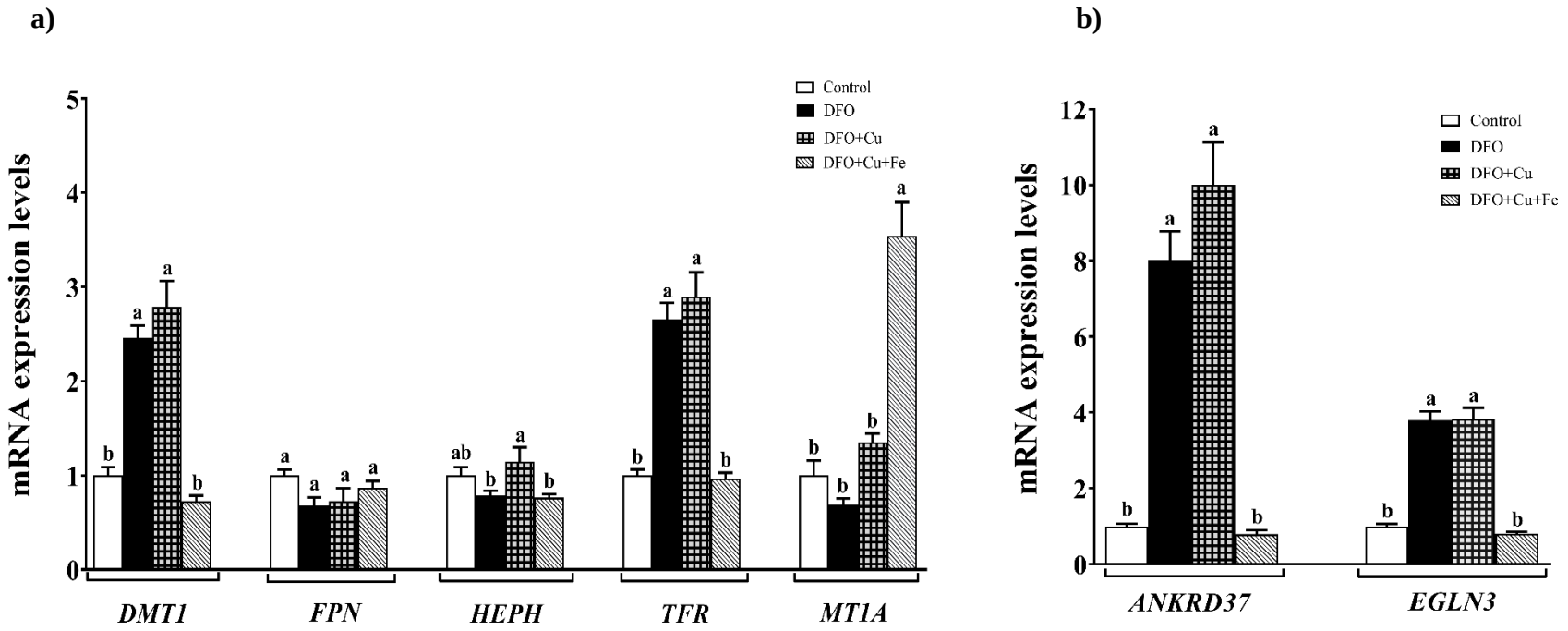


Figure 3.5. The effect of copper mineral on iron and hypoxia-regulated gene mRNA expression levels under the anemic condition in Caco-2 cells grown on the 12-well cell culture system for 21 days. The effects of Cu and Cu with Fe on iron (a) and hypoxia (b) regulating gene mRNA expressions. Data presented as mean ± SD (n = 3). Different letters on bars indicated significance ($p < 0.05$).

levels. Moreover, it was observed that Cu and Fe together significantly upregulated *MT1a* mRNA expression.

Enterocyte cells are unique cell types in the intestine due to the interaction between diet and the internal circulatory system of the body. Thus, the dietary and blood-derived stimulus might play a role in enterocyte nutrient metabolism. Copper and iron have physiological interactions in enterocyte cells during IDA. In many mammalian species, iron deficiency leads to elevated Cu levels in enterocytes (Ravia et al., 2005), serum (Yokoi et al., 1991), and liver (Sherman and Moran, 1984), indicating that copper might influence intestinal iron metabolism. Iron and copper interactions have been investigated in brindled mice ($Mo^{Br/y}$) and researchers observed no perturbation in iron absorption during anemia (Gulec and Collins, 2013). In this study, the enterocyte copper level of mutant mice was high due to nonfunctional ATP7 protein. In contrast, these mice were systemic copper-deficient; thus, enterocyte cells were exposed to two different copper levels. Furthermore, it was observed that dietary-induced iron deficiency upregulated mRNA expressions of iron-regulated genes, including *DMT1*, *TFR*, and *ATP7A*, but high enterocyte copper level did not affect the gene regulations suggesting that blood Cu level might be an important dietary factor for gene regulations in enterocyte cells during IDA. Thus, Caco-2 cells were grown on the bicameral cell culture plates to mimic the human intestine system to test whether dietary (apical side) or blood copper (basolateral side) are involved in iron-dependent genes regulation during IDA.

In the current study, marker genes that were regulated by iron deficiency anemia including iron transporters (*DMT1*, *TFR*, *HEPH*, and *FPN*) and iron-dependent hypoxic genes (*ANKRD37* and *EGLN3*) were selected. Furthermore, *MT1A* mRNA level was used to control intracellular mineral uptake since *MT1A* is regulated by intracellular Cu and Zn, respectively (Gefeller et al., 2015, Gulec and Collins, 2014). The results showed that Cu and Cu with Fe in the basolateral side of the cells reduced marker genes mRNA levels (*DMT1*, *FPN*, *TFR1*, *HEPH*, and *ANKRD37*) compared to the apical Cu and Cu with Fe treatment group during IDA. This suggested that Cu sensing of the basolateral side of the cells might be different from Cu sensing of the apical side. Cu regulates the *MT1A* mRNA levels, and its level is correlated with elevated intracellular Cu (Gulec and Collins, 2014). It was observed that *MT1A* mRNA levels were significantly induced when Cu and Cu with Fe were given to the basolateral side of the cells suggesting that the level of Cu in the blood might be a compelling factor for the regulation of iron deficiency-related genes in the intestine. Furthermore, the basolateral side of the enterocyte cells may play a role

in Cu sensing. The importance of the molecular and genetic interactions between basolateral and apical sides of polarized enterocyte cells has been indicated in different studies. It was shown that glucose treatment in the basolateral side of the Caco-2 cells induced apical cholesterol uptake and the mRNA levels of the cholesterol transporter gene (Ravid et al., 2008), indicating that basolateral signaling influences fatty acid metabolism through the apical side of enterocyte cells. Moreover, Han et al. (2002) showed that both apical and basolateral Cu treatment increased Fe uptake in non-anemic Caco-2 cells. Moreover, Cu treatment of apical and basolateral sides also induced *DMT1*, *TFR*, and *FPN* mRNA levels compared to the control group. All together results suggest that basolateral and apical sides of enterocyte cells might have a different physiological response regarding the same nutritional stimulus.

Caco-2 cells can differentiate on the regular tissue culture plate when they are grown 21 post-confluent days. Differentiation of Caco-2 cells gives different genetic responses for a variety of cellular physiological pathways (Sambuy et al., 2005). However, the polarization of those cells on the insert system might be another factor that can influence genetic regulation. Thus, Caco-2 cells were also grown on regular tissue culture plates at the same time that performed bicameral cell culture experiments. It was observed similar results for marker genes regulation between apical side Cu treatment groups and Cu treatment on Caco-2 cells that were grown on tissue culture plates. However, basolateral Cu treatment affected regulations of *DMT1*, *FPN*, *HEPH*, *MT1A*, *ANKRD37* genes compared to results from the cells that were grown on tissue culture plates. Our results suggested that the polarization of the Caco-2 cells might be an important factor for gene regulation in terms of Cu treatment in this study. It might be better to account for polarization of the Caco-2 cells for gene regulation or whole-genome array studies.

3.7. Conclusion

The nutrient-dependent regulation of enterocyte cells is central to the intestinal nutrient-sensing mechanism. The basolateral and apical sides of the enterocyte cells are the primary targets to understand nutrient sensing in terms of nutrient overload or deficiency. Furthermore, the polarization of Caco-2 cells also might influence gene regulation. The results obtained from studies of the last experimental chapter of this thesis

suggested that intracellular gene regulation was mainly affected by copper treatment in the basolateral side of enterocyte cells during IDA, indicating that blood copper level might have the ability to control the enterocyte iron metabolism at molecular and genetic levels during iron deficiency anemia. The blood copper level might be an important regulator for intestinal iron metabolism during iron deficiency anemia. The main application of this finding might be that copper mineral could be considered to add with iron mineral for functional food development processes or iron supplements to reduce the risk of iron deficiency.

CHAPTER 4

INVESTIGATION OF THE EFFECTS OF IRON MINERAL ON IRON METABOLISM UNDER ANEMIC CONDITIONS

Iron deficiency is the most common and widespread global nutritional disorder especially in women, infants, and children, and is defined as a condition in which there are no mobilizable iron stores and in which signs of a compromised supply of iron to tissues (WHO, 2001). 2 billion people, over 30% of the world's population, are anemic many due to iron deficiency. In developing countries, every second pregnant woman is estimated to be anemic that leads to adverse outcomes such as an increase in the risk of maternal mortality, perinatal mortality, and low birth weight. A nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from the diet. If iron intake is limited or inadequate due to poor dietary intake, or in the presence of some chronic diseases iron deficiency anemia may occur. Thus, the iron mineral that comes from the diet is important and the intestine plays a vital role to maintain iron homeostasis in the human body. However, whether dietary iron or blood iron level is determinant in the intestinal iron metabolism is unknown. Within this context, the objective of this chapter was to investigate the effects of dietary and blood iron levels on the molecular and genetic regulation of intestinal iron metabolism.

4.1. Cell Culture

The human colorectal adenocarcinoma epithelial cell line, Caco-2, was purchased from the American Type Culture Collection (ATCC, HTB-37, Manassas, VA). Caco-2 cells were cultured in minimal essential medium (MEM) (Sigma, United Kingdom) supplemented with 15% fetal bovine serum (FBS) (Gibco, Cat. No. 10500), 1% penicillin and streptomycin (100 U/mL) and, 1% nonessential amino acid solution (Gibco, Cat. No. 11140). They were maintained in 75 cm^2 culture flasks at 37°C in a constant humidified

incubator with an air atmosphere of 5% CO_2/95% O_2. When cultures reached 70-80% confluency, they were plated for either subsequent passage or treatment. Caco-2 cells used for the treatment experiments were between the 20^{th}-30^{th} passages.

4.2. Investigation of Importance of Dietary and Blood Iron on Intestinal Iron Deficiency

To investigate the iron metabolism, classical cell culture plates (12-well plates) were used whereas special bicameral insert systems (12-well inserts) were used to model the human small intestine system as the luminal region (apical part) in contact with the food and the region in contact with the blood circulation system (basolateral part).

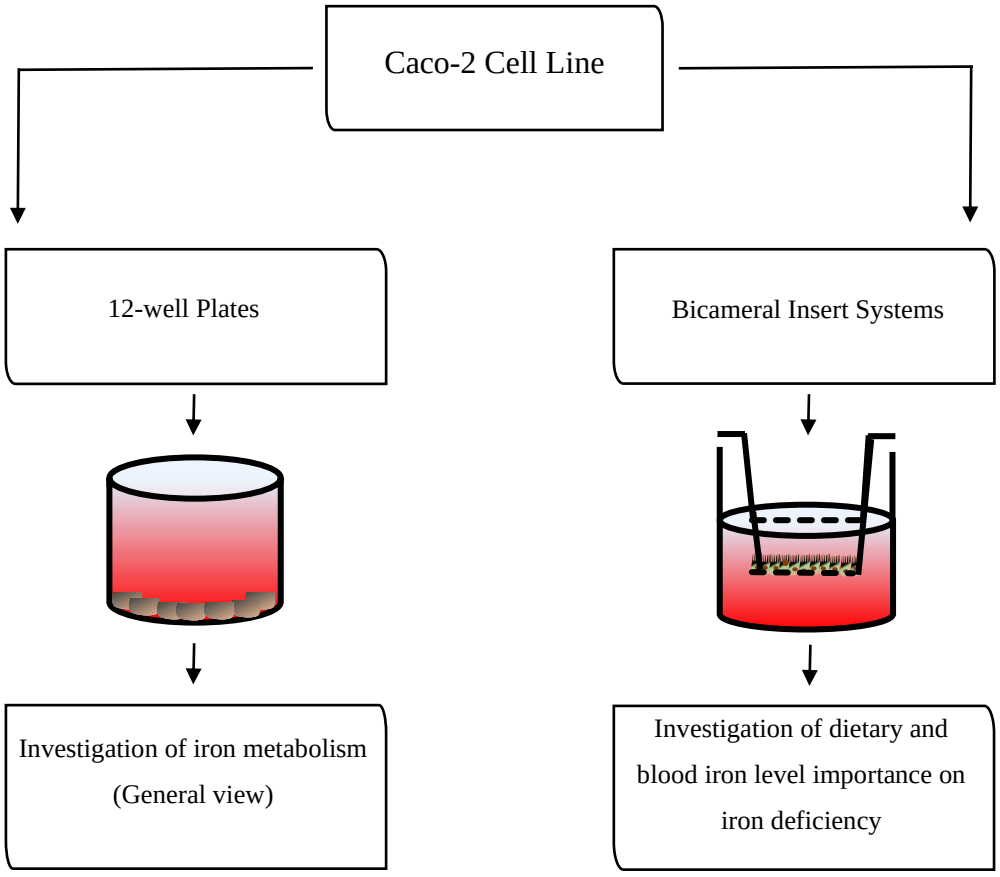


Figure 4.1. Diagram of the investigation experiments of iron mineral on iron metabolism under anemic conditions.

4.2.1. Determination of Cell Density on 12-well Plates

Cell seeding density which is one of the important culture-related factors, was standardized in order to make experiments reproducible and reliable. The cells were seeded at a density of 3 different levels: 6×10^4, 1×10^5, and 4×10^5 cells per cm^2 on polystyrene well plates (Corning) on standard conditions as described in Section 3.1 to form a monolayer (Figure 4.2). They were allowed to differentiate for 21 days prior to experiments. The medium was changed every 2-3 days.

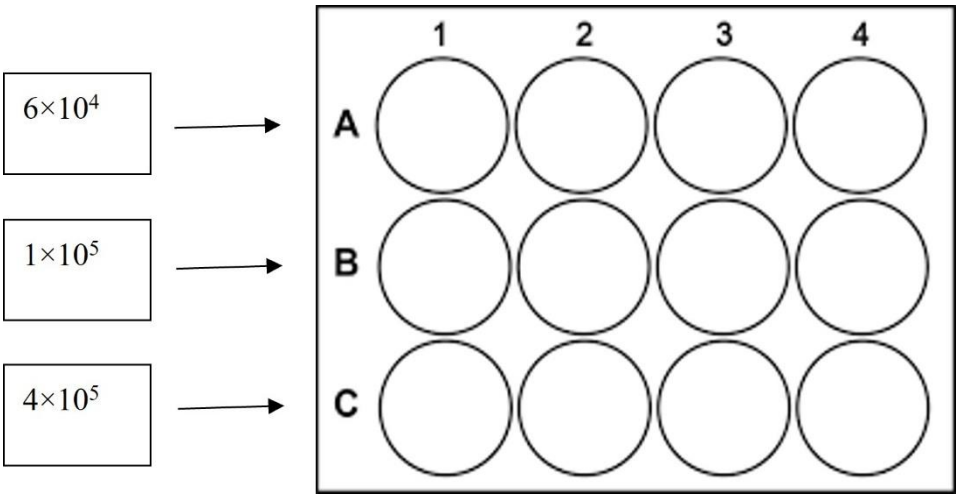


Figure 4.2. Cell density determination experiment on polystyrene 12-well plates.

4.2.2. Modeling of Human Intestinal System and Formation of Cell Barrier System

Caco-2 cell line models the human intestinal system by mimicking its physiological properties when grown for 21 days on special membranes. The Caco-2 cells were seeded in bicameral collagen-coated12-well inserts and grown to form a monolayer. Under standard conditions for 21 days, the cells were polarized. The bicameral system consisted of two parts: A 0.4 µm sized pore and a 12-mm diameter polytetrafluoroethylene membrane covered with collagen protein to help for cell adhesion (apical part), and a 12-well cell culture plate, which this membrane was placed in

(basolateral part) shown in Figure 4.3. The apical part was modeled as the part where the nutrients come in whereas the basolateral part was modeled as where the nutrients pass into the blood.

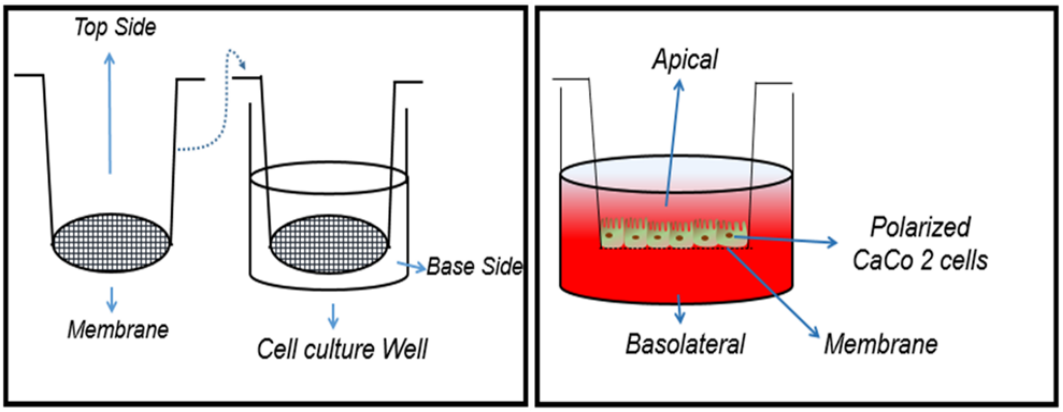


Figure 4.3. Special bicameral cell culture insert system.

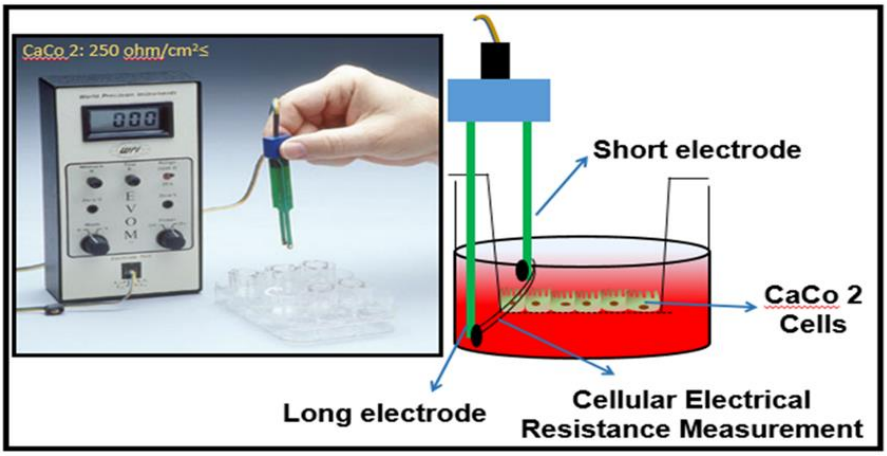


Figure 4.4. Measurement of the transepithelial electric barrier resistance (TEER).

Transepithelial electrical barrier resistance (TEER) was measured by an Epithelial Voltohmmeter (World Precision Instruments, Inc., FL, USA) to control if the cells were maintained their monolayer and polarized structure. The evommeter had two electrodes of different lengths (Figure 4.4). The shorter electrode was placed in the apical part whereas the longer electrode was placed in the basolateral part. The barrier resistance

created by the cells was measured by the device in ohms. It was stated that this value was at least 250 ohm/cm^2 for Caco-2 cells and it was noted that this value was reached after 4-day-cell incubation (Liang et al., 2000). It was very important that the cell barrier system was exactly at the desired level for further experiments.

4.2.3. Induction of Iron Deficiency and Iron Treatment

In order to investigate the effects of iron treatment on molecular and genetic regulation of iron metabolism on anemic cells, iron deficiency anemia was induced using a chemical agent (Deferoxamine, DFO), which bound available iron to DFO. The formed iron-DFO complex was not available for the cells anymore. When the cells reached 21-day-confluency in 12-well plates and 12-well- inserts with the determined density, DFO was added into the medium (200 µM) for 24h to develop anemic conditions. After 24h, anemic cells were treated with iron (FAC, ferric ammonium citrate; 100 µg/mL) for 18h (Figure 4.5).

The experimental groups used for the determination of the effects of iron mineral on iron metabolism in anemic cells growing on 12-well plates and 12-well inserts were shown in Figure 4.6 and Figure 4.7., respectively.

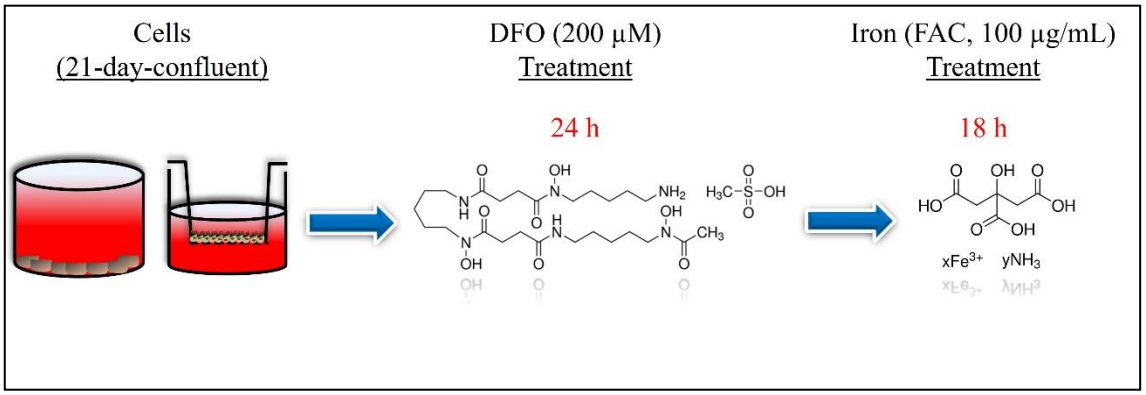


Figure 4.5. Induction of iron deficiency and subsequent iron treatment in cells growing on 12-well plates and 12-well inserts.

4.3. Quantitative Real-Time Polymerase Chain Reaction

The mRNA expression levels of the marker genes that regulate iron and hypoxia were evaluated. Total RNA was isolated from cells with RNAzol reagent (MRC, Cat. No.: RN190) following to manufacturer's protocol. One microgram of each total RNA was converted to cDNA using the cDNA synthesis kit (Lifetech, Cat. No.: 4368814). qRT-PCR was performed on an ABI StepOnePlus instrument (Lifetech, CA, USA) by using gene-specific oligonucleotide primers and SYBR-Green mix (Lifetech, Cat. No.: 4367659). C_T (threshold cycle) levels were normalized according to human cyclophilin A (CypA) mRNA expression as a housekeeping gene. Mean fold changes in gene-specific mRNA levels from all experimental groups were calculated by the $2^{-\Delta\Delta Ct}$ analysis method (Gulec and Collins, 2013).

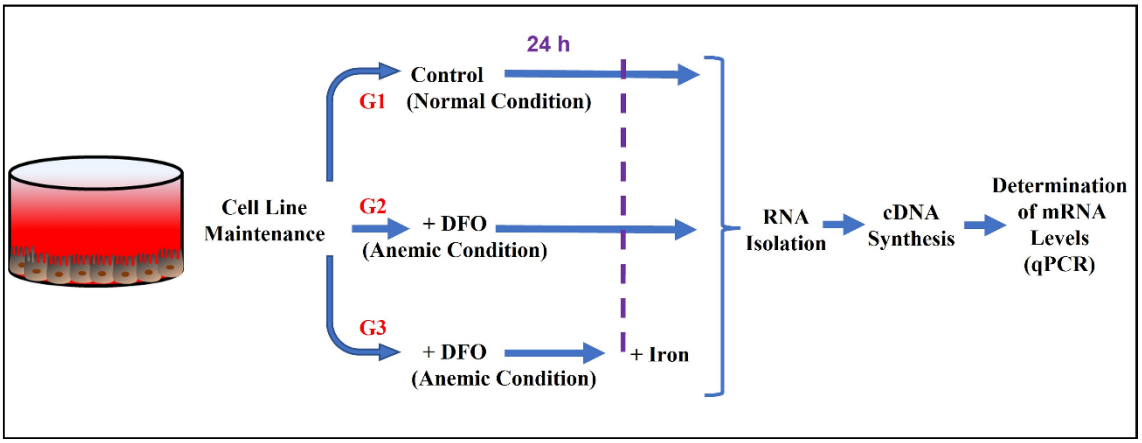


Figure 4.6. Determination of the effect of iron mineral on the molecular and genetic regulation of iron metabolism in anemic cells growing on 12-well plates. G1: Control group, G2: Anemic group, G3: Iron treated group in anemic condition.

4.4. Statistical Analysis

Statistical analysis for more than two groups was carried out by using "One-way analysis of variance (ANOVA)" with Tukey's posthoc test (significance threshold of $p \leq$

0.05). PRISM software, version 6 (Graph Pad Software, Inc., San Diego, CA, USA) was used in the analysis of statistics and constructing the figures. Results were expressed as mean ± SD of triplicate experiments and considered statistically significant for *p* values less than 0.05.

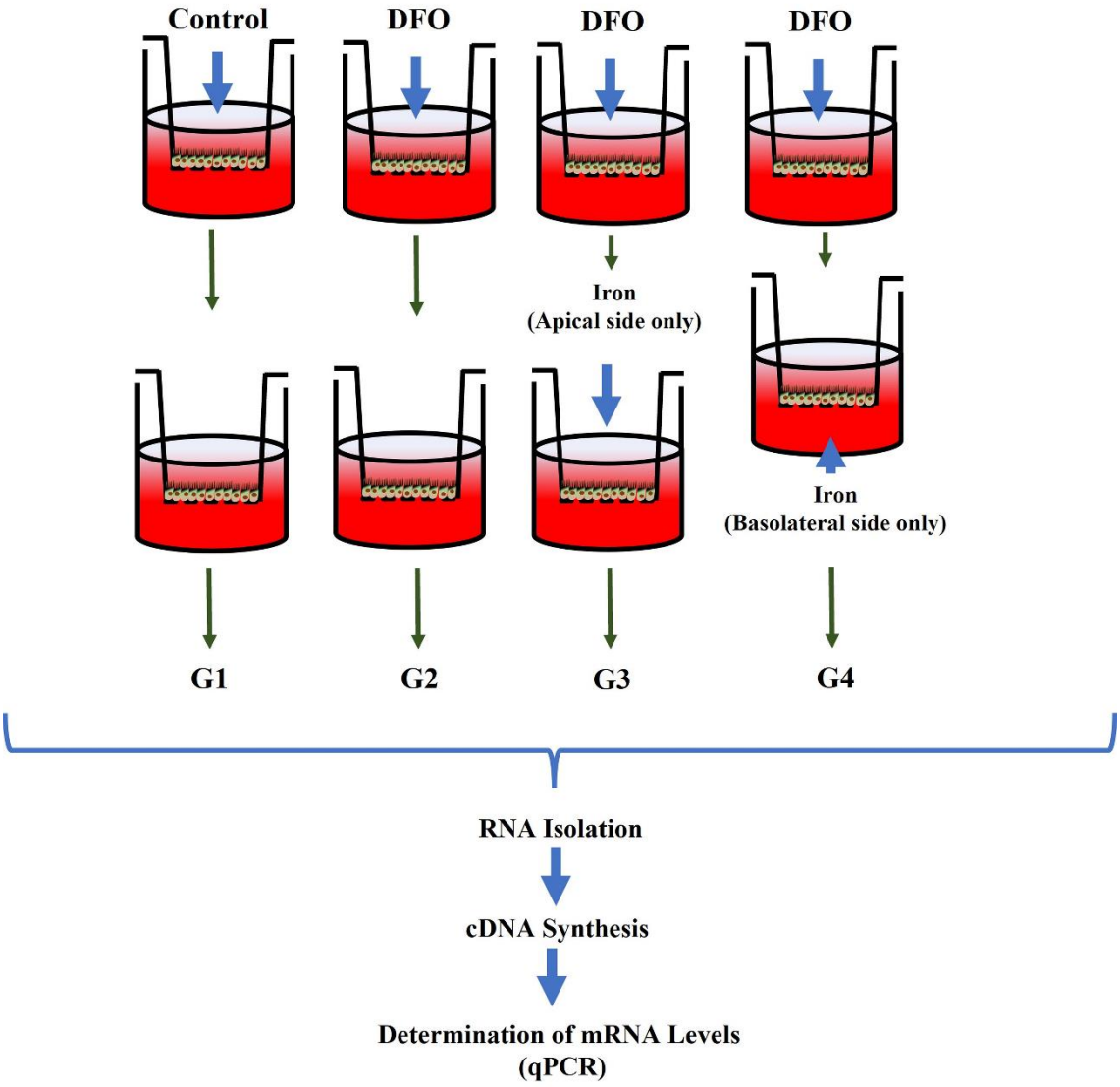


Figure 4.7. Determination of the effect of the iron mineral from the diet or from the diet on polarized anemic cells growing on 12-well inserts. G1: Control group, G2: Anemic group, G3: Apical side-iron treated anemic group, G4: Basolateral side-iron treated anemic group.

Table 4.1. Marker genes that were used in the evaluation of the effect of treatments on iron metabolism.

Iron Metabolism	Hypoxia
DMT1	*ANKRD37*
FPN	*EGLN3*
FTN	
HEPH	
TFR	

4.5. Results and Discussion

4.5.1. Caco-2 Cell Line and Effect of Cell Density on Cell Culture

Due to the hardness of ex vivo use of enterocyte cells from intestinal tissues in a short period of time, alternative cell models that are cultivable and metabolically active under appropriate conditions have gained importance. Considering the accessible cell models, normal human enterocyte cells are rarely encountered in the literature. Therefore, it is important to use cells that can be closely mimic the human intestine system. Caco-2 is a well-characterized human colon adenocarcinoma cell line, and it has been extensively used as the intestinal barrier for over forty years. The cells undergo spontaneous differentiation when grown on a bicameral cell culture insert system for about 14 to 21 days that giving great similarity to human polarized absorptive enterocyte cells in terms of functional, metabolic, and biological properties (Sambuy et al., 2005). Compared with other colon carcinoma cell lines, the CaCo-2 cell line has been shown to have better morphological and functional properties for nutritional metabolism studies (Chantret et al., 1988). Caco-2 cell line model is commonly used for investigating the enterocyte iron and copper metabolisms (Han and Wessling-Resnick, 2002; Linder et al., 2003; Chicault et al., 2006; Zhu et al., 2006; Pourvali et al., 2012)., transport of nutrients (Yin et al., 2014), drugs (Sevin et al., 2013) and functional substances (Satake et al., 2002). For all reasons above, the Caco-2 cell line was chosen for the experiment of this thesis.

There are some culture-related factors that should be strictly standardized in order to make experiments reproducible and reliable. Cell seeding density is one of the important culture-related factors considering that cells undergo differentiation when they reach confluence. Different studies have been reported a wide variety of cell seeding densities ranging from 2×10^4 (Dalmasso et al.,2010) or 6×10^5 cells/cm^2 (Yu and Hung, 2013). In this study, 3 different levels (6×10^4, 1×10^5, and 4×10^5 cells/cm^2) of cell seeding density were screened on polystyrene 12-well plates. At the end of the second day of the cell seeding experiment, it was observed that the wells inoculated with lower cell density (6×10^4 cells/cm^2) were not covered by cells, the wells inoculated with medium cell density (1×10^5 cells/cm^2) were confluent with the monolayer. The wells inoculated with high cell density (4×10^5 cells/cm^2) also reached confluency, however, they formed the multilayer structure which caused cell detachment. It can be thought that higher seeding densities of cells could be advantageous to reach confluency in a shorter time, nevertheless as figured out in the experiment due to the cell detachment it is most likely that the cells will end up with death. As a result, cell density for further experiments was determined as 1×10^5 cells/cm^2 in accordance with the other studies in the literature (Senarathna and Crowe, 2015; Span et al., 2016).

4.5.2. The Effect of Iron Treatment on Molecular and Genetic Regulation of Iron Metabolism of the Cells Growing on 12-well Plates

The effects of iron mineral on iron metabolisms of on anemic cells grown on 12-well plates were evaluated in terms of molecular and genetic regulation. According to the results (Figure 4.8), when DFO was given to the cells and anemia induced expression levels of *TFR* and *DMT1* genes increased, whereas the expression levels of *FPN*, *FTN*, and *HEPH* genes decreased significantly. Also, it was also understood from the figure after iron treatment on anemic cells, the expression levels of all marker genes showed similar profiles with the control group, and they were not different statistically. In other words, iron treatment was efficient to recover anemic cells.

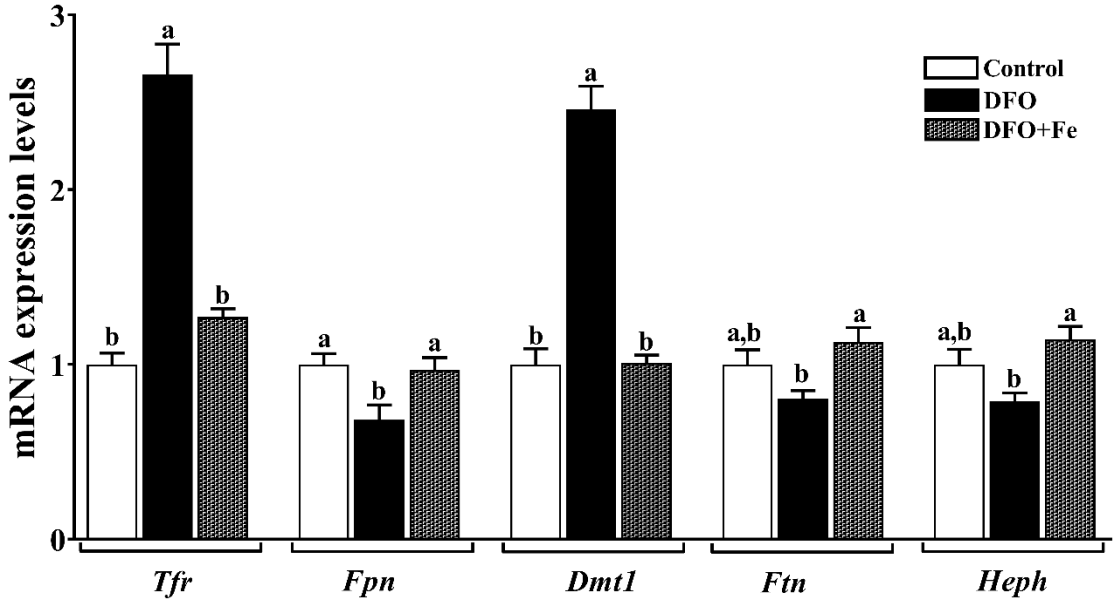


Figure 4.8. The effect of iron treatment on iron metabolism on anemic cells growing on 12-well plates. *Tfr*: Transferrin Receptor, *Fpn*: Ferroportin, *Dmt1*: Divalent metal transporter 1, *Ftn*: Ferritin, *Heph*: Hephaestin. Data presented as mean ± SD (n = 3). Different letters on bars indicated significance ($p < 0.05$).

Hypoxia is integrally related to iron metabolism. Intestinal hypoxia-inducible factor 2α (HIF-2α), which is a transcriptional factor, is essential for iron absorption during iron deficiency anemia by regulating apical and basolateral iron transporters (Schwartz et al., 2019). Iron regulates the hypoxic response and hypoxia alters levels of iron regulatory and storage proteins (Harned et al., 2014**).** mRNA regulation of Ankyrin repeat domain 37 (*ANKRD3*) and Hif prolyl hydroxylase 3 (*EGLN3*) were the most well-known marker genes for the hypoxic signal under iron deficiency. These marker genes were investigated in order to understand that how the hypoxic signal changed when the iron was given to cells. As illustrated in Figure 3.9, hypoxia due to iron deficiency anemia increased the expression of *ANKRD37* and *EGLN3* mRNA by about 8- and 4-fold, respectively. According to the study conducted by Hu et al. (2010), it was demonstrated that iron mineral recovers an increasing intracellular hypoxic environment. In this study, the iron supplied to the anemic cells decreased the expression level of *ANKRD37* and *EGLN3* mRNA significantly to the expression level of the mRNA of the control group in accordance with the literature.

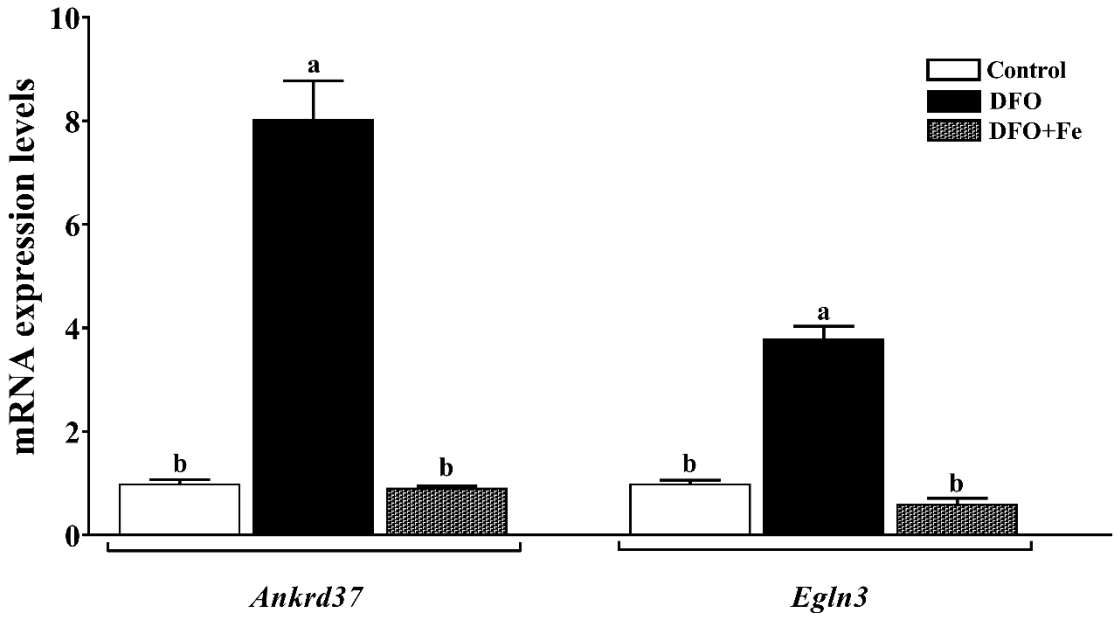
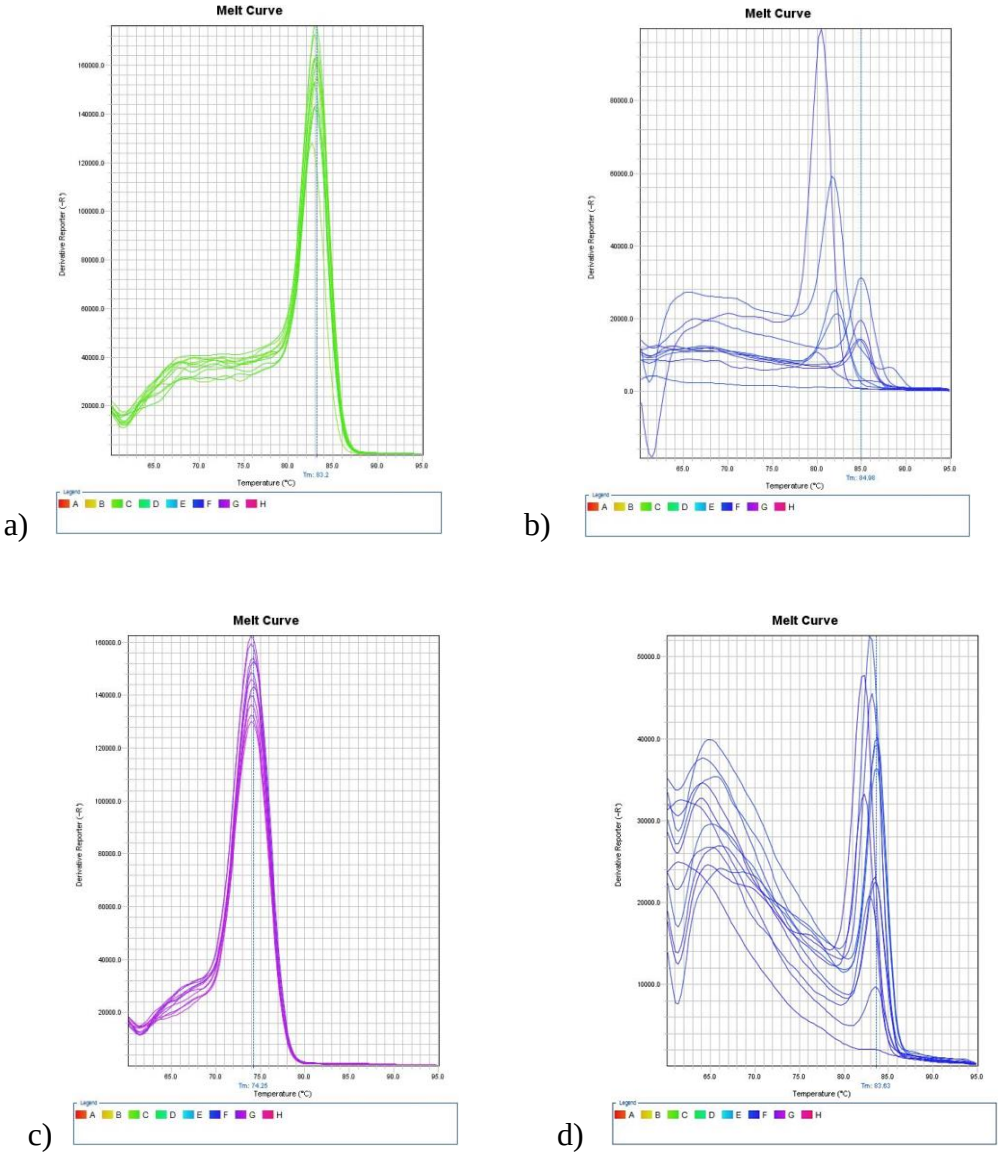


Figure 4.9. The effect of iron treatment on hypoxia-induced gene expression on anemic cells growing on 12-well plates. *ANKRD37*: Ankyrin Repeat Domain 37, *EGLN3*: Hif prolyl hydroxylase 3. Data presented as mean ± SD (n = 3). Different letters on bars indicated significance ($p < 0.05$).

"Melting Curve Analysis" was used to control whether the primers recognized the target mRNAs in the qPCR protocol, or they bound to other non-specific sequences. This step was controlled for each primer and each qPCR assay performed in the study. The melting curves of some genes were illustrated in Figure 4.10. The uniform peaks seen in the figure indicated that the primers were specifically bound to the target sequences. However, it was encountered with some problems when the 'Melting Curve' analysis was applied for *DCYTB*, and *P4HA1* primers, the genes planned to be studied within the scope of the thesis. The experiments were repeated with the primers re-ordered to different companies, nevertheless, primers continued to bound non-specific sequences. Hence these two marker genes were excluded from the study.



a) b) c) d)

Figure 4.10. 'Melting Curve' results from selected primers. A) Melting curve of *EGLN3*, b) melting curve of *P4HA1*, c) melting curve of *FPN*, d) melting curve of *DCYTB*.

4.5.3. Effect of Iron Treatment on Molecular and Genetic Regulation of Iron Metabolism of the Cells in Bicameral System (Modeling of Human Intestinal System)

Caco-2 cell line models the human intestinal system by mimicking the physiological properties, namely they differentiate to polarize, generate microvilli on the

apical side of the cell membrane and form tight junctions between adjacent cells of enterocyte cells responsible for absorption when grown for 21 days on special membranes (Alvarez-Hernandez et al., 1991, Yu and Huang, 2013).

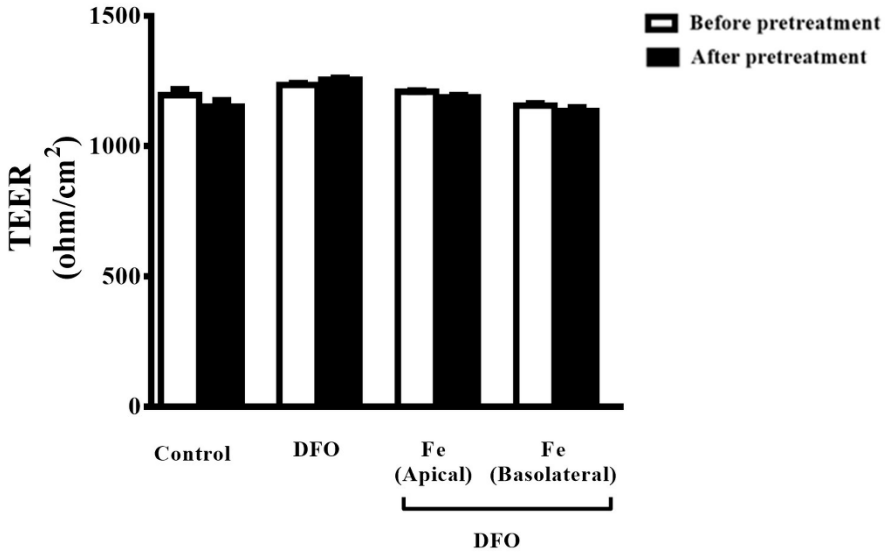


Figure 4.11. Determination of cell polarization by TEER and effect of experimental treatments on cell barrier resistance. Data presented as mean ± SD (n = 3). Different letters on bars indicated significance ($p < 0.05$).

The polarization of Caco-2 cells was checked by measuring cell barrier resistance (Transepithelial electric resistance, TEER) across the cell monolayer. TEER was measured at the end of 21-day-incubation in order to control cell differentiation. Similarly, after DFO and iron treatments to the cells, TEER was also checked in order to control whether the treatments affected the cell structure and viability. According to the measurements, there was no significant change in TEER values before and after cell treatments among the experimental groups as shown in Figure 4.11. Therefore, it could be concluded that DFO and iron given to the cells were taken only paracellular way.

Subsequently, the effect of iron minerals that came from the diet or found in the blood on polarized anemic cells was investigated. After checking the TEER values of the 21-day-confluent cells, DFO and iron treatments for the specified amounts and duration described as in Section 4.2.3 were given to the cells. Thereafter, as described in Section

4.3, RNA isolation, cDNA synthesis, and subsequent qPCR method for the target genes indicated in Table 4.1 were performed. The cells were grown in the bicameral system in order to model the physiological structure of the human intestinal system. The effect of dietary and blood iron mineral level on enterocyte iron metabolism and hypoxia under anemic conditions was investigated by giving iron minerals to apical and basolateral parts of the system, separately. The apical part represented the part where the nutrients come in whereas the basolateral part represented the part where the nutrients pass into the blood. It could be considered that it was very difficult to study the effects of dietary and blood iron levels separately in animal studies because of the systemic relationship between organs and systems in the human body. The *in vitro* cell model used in this study enabled us to determine the effects of iron minerals that came from nutrients or found in blood on enterocyte cells, separately.

First, the effect of iron minerals on iron metabolism was investigated (Figure 4.12). It was observed that *TFR* and *DMT1* mRNA expression levels increased under iron deficiency induced by DFO compared to the control group. Subsequent reduction of dietary nonheme ferric iron, Dmt1 transports the ferrous iron from the absorptive surface of the small intestine across the apical membrane of enterocytes (Gulec et al., 2014). The results were in accordance with the previous studies in which it had been reported that the intestinal *DMT1* expression had been strongly upregulated by iron deprivation and consequent hypoxia, accounting for the increased uptake of iron under in deficiency (Linder et al., 2003; Gulec et al., 2014). Transferrin is the plasma iron transport protein and Tfr-bound ferric iron is distributed to the circulation throughout the body (Gulec et al., 2014). It had been shown that Tfr expression was increased in response to iron deficiency and decreased when intracellular iron levels were high (Zoller et al., 2002). Therefore, it could be said that an increase in TFR1 expression was indicative of iron deficiency as observed in the experiments. As illustrated in Figure 4.12, apical iron mineral significantly increased mRNA expression of Heph protein compared to the control group. On the other hand, when the iron was applied to the basolateral side, the level of *HEPH* mRNA decreased significantly compared to all experimental groups. In addition, this decrease was down to the level of the gene expression level in the control group. The membrane-bound ferroxidase Heph is believed to facilitate iron out of the cell by oxidizing the soluble ferrous iron into the ferric state prior to its release by Fpn which is the only known iron exporter in humans (Lee et al., 2012; Vashchenko and MacGillivray, 2013). According to the results obtained, an increase in *FPN* expression

was found upon DFO treatment of Caco-2 cells in the case of apical iron treatment, whereas *FPN* expression was reduced upon basolateral iron treatment. A regulated localization process for hephaestin in response to apical iron could provide the means to regulate iron export only when dietary iron is available and prevent depletion of cellular stores (Lee et al., 2012). Ftn is the well-known marker for cellular iron uptake and stores iron when not needed elsewhere in the organism. As seen in Figure 4.12, mRNA levels of *FTN* did not differ between the experimental groups.

It has been shown that when DFO was given to Caco-2 cells the level of Hif2α protein increased and this increase caused an increase in Ankrd37 and Egln3 levels (Hu et al., 2010). Hypoxic condition diminished due to iron mineral given to the cells. In this study, the iron mineral that had been administered to the cells reduced the levels of *ANKRD37* and *EGLN3* mRNA to levels in the control group shown in Figure 4.13. In other words, hypoxia due to iron deficiency anemia increased the expression of *ANKRD37* and *EGLN3* mRNA compared to control groups. Also, it was observed that there was no significant difference between the mRNA levels of these genes when the iron was treated apically and their levels of DFO. In contrast, basolateral iron treatment of cells significantly reduced these protein expressions. These results suggested that the iron mineral level in the blood could be more important in the regulation of hypoxic response in enterocyte cells.

4.6. Conclusion

The Caco-2 cells were grown in classical cell culture plates (12-well plates) and special bicameral cell culture insert systems for 21 days and investigated the effects of iron mineral on iron metabolism and hypoxia in case of iron deficiency anemia. It is unknown that whether dietary iron or blood iron level is determinant in the intestinal iron metabolism to maintain iron homeostasis in the human body. So, one of the most important parts of this chapter was the modeling of the human small intestine system. In this way, the human body was modeled as the luminal region (apical) in contact with the food and the region in contact with the blood circulation system (basolateral). This polarization allowed us to investigate the molecular and genetic effects of iron minerals

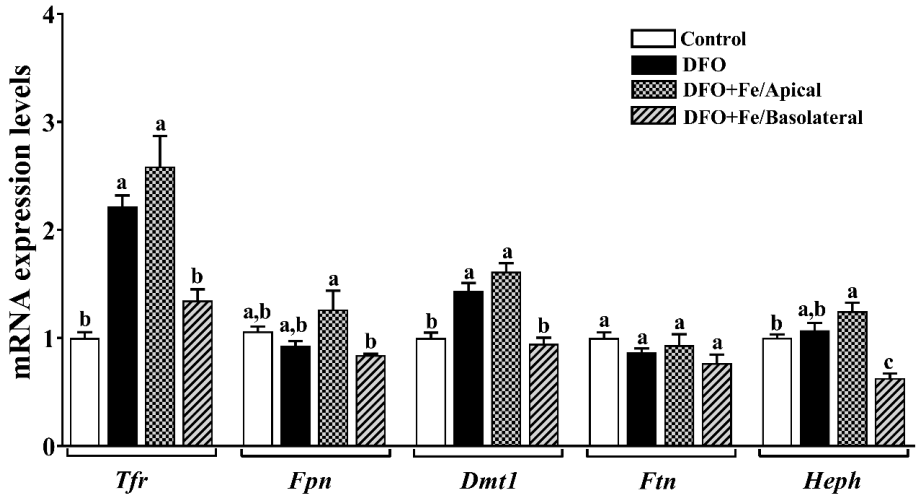


Figure 4.12. The effect of iron treatment on iron metabolism on anemic cells growing on 12-well inserts. *TFR*: Transferrin Receptor, *FPN:* Ferroportin, *DMT1:* Divalent metal transporter 1, *FTN:* Ferritin, *HEPH:* Hephaestin. Data presented as mean ± SD (n = 3). Different letters on bars indicated significance ($p < 0.05$).

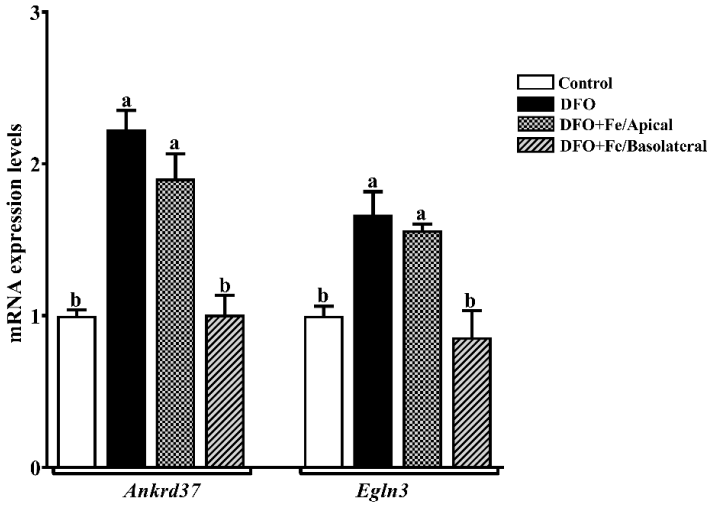


Figure 4.13. The effect of iron treatment on hypoxia-induced gene expression on anemic cells growing on 12-well inserts. *Ankrd37:* Ankyrin Repeat Domain 37, *Egln3:* Hif prolyl hydroxylase 3. Data presented as mean ± SD (n = 3). Different letters on bars indicated significance ($p < 0.05$)

found in the blood and/or come from diet separately on anemia and anemia-dependent hypoxia in the enterocyte cells of the human small intestine. When comparing the levels of DFO and iron-treated cells grown on plates to cells grown on inserts, it was observed that there was a significant difference in the regulation of *DMT1*, *FTN*, and *HEPH* genes. The main reason for this change was thought to be due to differences in the way cells growth, in other words, due to the localization of proteins. For example, in human enterocyte cells, Dmt1 protein functions in the apical region whereas Fpn protein functions in the basolateral region. Also, apical and basolateral affects Heph localization in polarized cells. Since there was no polarization in plates it was not possible to predict such a difference, the localization and regulation of the proteins might be similar to the in vivo situation due to polarization in the cells grown on the membrane. Also for the cells grown on inserts, the most important point out of these results was that the iron mineral given to the basolateral part (the part of the enterocyte cell which is facing the blood) showed the significant effects on the genes that play a role in iron metabolism compared to the apical part (the part that in contact with the food). So, it can be concluded that the level of iron in the blood might be more important than the dietary intake. However, the most important point that should not be missed was that blood iron level was regulated with dietary iron. There are three ways to maintain iron homeostasis in the human body:

(i) iron can be injected into one of the blood vessels,

(ii) iron pills can be used as supplements or,

(iii) by the development and consumption of functional foods that have high iron bioavailability.

Frequent injection of iron is inconvenient and considering the toxicity of iron, it can cause administration problems. Also, some problems that come from taking pills are common such as their side effects (constipation, diarrhea, sickness, vomiting, etc.) and swallowing pills can be a problem, either. Therefore, it is important to develop edible natural iron supplements that can be used as an additive to food products to make them “functional” via enhancing their iron bioavailability in order to reduce iron deficiency risk in humans. With this study, it was shown that the mechanism of why we need to develop a functional food.

CHAPTER 5

FUNCTIONAL PROPERTIES OF LEGUME PROTEINS AND IRON SALT

Protein functionality could be a useful instrument for both industry and human wellbeing. Proteins with mineral chelating activity have been shown to have potential applications in the treatment of mineral deficiencies. Several research studies have examined the chelating ability of proteins and/or peptides to promote and enhance mineral bioavailability. Hence, chelating proteins and/or peptides, isolated from vegetable protein hydrolysates, may also improve the bioavailability of iron minerals without causing any side impacts on human wellbeing.

Legumes are a low-cost source of protein that are also beneficial for human health. They also have ability to chelate iron minerals. From a nutritional standpoint, the iron-chelating ability of legume proteins is of importance since they may have high iron mineral stability against *in vitro* digestion because they are bound to proteins.

This chapter describes the functional properties of legume proteins in terms of total protein and water-soluble protein amounts and iron chelating abilities at various pH conditions. Moreover, iron salt solubility was evaluated and compared to protein solubility profiles to find out the optimum binding conditions for further protein-iron complexation experiments which are one of the subjects of Chapter 6. Finally, iron chelating capacities of protein extracts from all legumes were investigated.

5.1. Materials

The dried chickpeas (cv. Koçbaşı) and red lentils were purchased from a supermarket in Izmir, Turkey, and dried soybeans (non-GMO) were ordered from a local company. They were refrigerated until used. Peas were purchased from a local market in İzmir and frozen until used. Ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), bovine serum

albumin (CAS: 9048-46-8), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) (CAS: 7782-63-0), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2), glacial acetic acid (CH_3CO_2H) (CAS: 64-19-7), hydrochloric acid (HCl) (CAS: 7647-01-0), sulfuric acid (H_2SO_4) (CAS:7664-93-9), orthophosphoric acid 85% (H_3PO_4) (CAS: 7664-38-2), ethanol 96% (CH_3CH_2OH) (CAS:64-17-5), sodium hydroxide (NaOH) (CAS:1310-73-2), and Coomassie® Brilliant blue G-250 (CAS:6104-58-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid monosodium salt (Ferrozine) (CAS: 69898-45-9) was purchased from Fluka (USA).

5.2. Protein Extraction

The crude protein extracts from legumes (lentil, chickpea, soybean, and pea) were prepared by the alkaline extraction method (Aydemir and Yemenicioglu, 2013). Briefly, 50 g of each legume were rehydrated overnight at 4°C in 500 mL of deionized water, separately. The mixtures were then homogenized for 2-4 minutes in a Waring blender, and the pHs were adjusted to 9.5 using 1 mol/L NaOH. Magnetic stirring was used to extract the homogenates for 2 hours at room temperature. The tough particles of insoluble debris were subsequently filtering out using cheesecloth, and the remained solution was utilised in protein purification by classical isoelectric precipitation (IEP). The protein extracts of all legumes were first clarified by centrifugation for 30 minutes at 15,000 g at 4°C. The proteins in the obtained supernatants were then precipitated by the IEP by changing the pH of extracts to 4.5 using 1 mol/L acetic acid. Centrifugation was used to collect the precipitated proteins, which were then resuspended in distilled water at pH 7.0. The IEP was then applied for the second time as described above, and the precipitated proteins were lyophilized following suspending in distilled water (Labconco, FreeZone, 6 L, Kansas City, MO, USA). The lyophilized legume protein extracts were obtained by the IEP method contain mainly globulins. The lyophilized chickpea, pea, lentil, and soybean protein extracts were coded as CPE, PPE, LPE, and SPE, respectively and stored at -20°C for several months until they were used for further experiments.

5.3. Total (TPrC) and Water-Soluble Protein Contents (WSPC) of Legume Extracts

The Kjeldahl method was used for the determination of total nitrogenous compounds in protein extracts of legumes by using an automated testing machine (Gerhard vapodest 50s and Kjeldahl Therm, Germany). 1 g of lyophilized protein extract of each legume was heated to 450°C using 25 mL of 96% (v/v) H_2SO_4, a catalyzer and antifoaming agent and maintained for 4 hours. After the burning step, the samples were cooled to room temperature and the titration process was carried out using 0.1M HCl. The total protein contents (TPrC) were calculated by using the conversion factor of 6.25. The average of three replicates was used to calculate the total protein contents and results were expressed as g protein per g of protein extract (g/g).

The water-soluble protein contents (WSPC) of protein extracts of each legume were determined by the Bradford method with minor modifications using bovine serum albumin (BSA) as standard (Bradford, 1976) (Appendix A) and the method was also adapted for small volumes (μL order) to reduce the amount of protein isolates and Bradford solution used. For the preparation of Bradford solution 100 mg of Coomassie Brilliant Blue, 50 mL of ethanol, and 100 mL of orthophosphoric acid (85 %) were dissolved in deionized water (total volume 1 L) and were filtered through filter paper. 20 mg of lyophilized protein extracts were dissolved in 10 mL deionized water at different pHs changing between pH 2.0 and pH 8.0 by using 0.01 mol/L NaOH or 0.01 mol/L acetic acid solutions. The suspensions were magnetically stirred for 30 min at room temperature, and they were centrifuged at 4500 g at 4°C for 20 min to remove insoluble residues. 5 μL of protein solution was added into 250 μL of Bradford solution and incubated for 5 min. The absorbance measurements of solutions were conducted at 595 nm. The protein analysis of each sample was run with three replicates and results were expressed as g soluble protein per g of protein extract (g/g) and also the percent solubility was calculated as the percentage ratio of protein in the supernatant to that of the total protein in the initial sample. The solubility profile was obtained by plotting the average protein solubility (%) against pH values.

5.4. Determination of Ferrous Iron Amount and Ferrous Iron-Chelating Ability (FIC) of Protein Extracts of Legumes

The ferrous iron amount was determined according to the method of Ward and Legako (2017) with minor modifications. Beer's Law describes the link between the absorbance of the chromogen which is ferrozine reagent and mineral combination. In this procedure, a standard curve is constructed with a stock iron solution to quantify the iron mineral in protein extracts of legumes. Briefly, 100 µL of each protein extract sample were mixed with 250 µL of 1mM ferrozine in a microplate. The solutions were well mixed and allowed to stand for 10 min-incubation at room temperature. After incubation, the absorbance was read at 562 nm with a microplate reader. Distilled water (100 µL) instead of sample solution was used as a blank. Sample solutions were used as color blanks, if necessary, which were used for error correction because of the unequal color of the sample solutions. The iron analysis of each sample was conducted with three replicates and results were expressed as µg/mL (Appendix B).

The ferrous iron-chelating ability of protein extracts of legumes was determined according to the method of Wang et al. (2009) before and after the gastrointestinal digestion with minor modifications. Briefly, 100 µL of each protein extract stock solution (5 mg/mL) were mixed with 135 µL of distilled water and 5 µL of 2 mM $FeSO_4.7H_2O$ in a microplate. The reaction was initiated by the addition of 10 µL of 5 mM ferrozine. The solutions were well mixed and allowed to stand for 10 min-incubation at room temperature. After incubation, the absorbance was read at 562 nm with a microplate reader. Distilled water (100 µL) instead of sample solution was used as a blank. Sample solutions (250 µL) were used as color blanks, if necessary, which were used for error correction because of the unequal color of the sample solutions. EDTA-Na2, $FeCl_2.4H_2O$, $FeSO_4.7H_2O$ were used as reference standards. The formation of violet color indicated weak chelating ability whereas the lack of any violet color development showed the strong iron binding. All measurements were performed in triplicate. The ferrous ion-chelating ability was calculated by using the following formula:

$$\text{Ferrous ion-chelating ability (\%)} = [A0 - (A1 - A2)] / A0 \times 100. \quad (4.1)$$

where A0 was the absorbance of the blank, A1 was the absorbance of the treated sample or standard and A2 was the original absorbance of the untreated sample.

5.5. Determination of Iron Solubility at Various pH Conditions

The iron salt, $FeSO_4.7H_2O$ was dispersed in water (1000 µg/mL), and the pH was adjusted using 1 mol/L NaOH or HCl to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, or 8.0 to obtain the solubility profile of iron. The dispersions were kept at room temperature (25 ± 2°C) with stirring (100 rpm) for 30 min and centrifuged (4500 g, 20 min, 4°C). The centrifugation force was the same as in the assessment of protein determination and bioaccessibility to be able to compare the chemical and physiological solubility profiles of iron. The iron content in the supernatants was determined by ferrozine assay as discussed previously in Section 5.4.

5.6. Statistical Analysis

Data were statistically evaluated by one-way analysis of variance (ANOVA) using the PRISM software, version 3.02 (Graph Pad Software, Inc., San Diego, CA, USA). Significant differences between mean values of results were determined by Tukey's Multiple Comparison Test procedure at the 5% significance level. Design Expert Version 11 was used for the statistical experimental design for all the simulated gastrointestinal digestion experiments with the response as soluble protein content (g soluble protein/g of protein extract). The results were evaluated statistically significant for *p* values less than 0.05.

5.7. Results and Discussions

5.7.1. Functional Properties of Legume Proteins

5.7.1.1. TPrC, WSPC, and pH-dependant Solubility Profiles of Lyophilized Legume Protein Extracts and Iron Salt

On a dry weight basis, legume seeds contain approximately 17-30% protein with varied concentrations of essential amino acids (Boye et al., 2010a). Although these percentages may change based on the plant growing conditions, species, and maturity; high molecular-weight storage proteins make up the majority of the protein present in legume seeds. Globulins are the principal storage proteins in legume seeds, accounting for 35-72% of total solubilized proteins among the four main classes of proteins found in legumes. It has been reported in the study conducted by Ghumman et al. (2016), globulins had superior digestibility compared to the other primary types of legume proteins. Protein availability is measured by digestibility. Highly digestible proteins are preferable from a nutritional standpoint because they supply more amino acids for absorption after digestion than proteins that have low digestibility. Also, the method used in this study, alkaline extraction followed by isoelectric precipitation, is the most prevalent way for preparing protein isolates in the food industry, therefore protein isolates rich in the globulin fraction would also be generated using this method (Kaur and Singh, 2007; Barbana and Boye, 2013, Jarpa-Parra et al., 2014). So, the proteins discussed in this thesis are mainly globulins.

Some functional properties of legume protein extracts are shown in Table 5.1. The total protein contents of legumes varied between 0.60 and 0.86g/g. The TPrC of lentils was found as the highest and the value was almost 1.4-fold higher than that of chickpea and soybean extracts, 1.2-fold higher than that of pea extract. TPrC of chickpea and soybean were lower than lentil, but similar with each other whereas pea extract was slightly higher than that of lentil.

Table 5.1. Total protein content, water-soluble protein content, and ferrous ion-chelating ability protein extracts of legumes

Protein Extract	**TPrC[a] (g/g)[b]**	**WSPC[a] (g/g)[b]**	**FIC (at a concent. 1500 µg/rxn. mix)**
Chickpea	0.60±0.02c	0.28±0.03b	55%
Lentil	0.86±0.02a	0.63±0.08a	69%
Pea	0.75±0.00b	0.37±0.01b	45%
Soybean	0.62±0.02c	0.29±0.04b	39%

[a] TPrC: Total protein content, WSPC: Water-soluble content

[b] Different letters in columns indicate statistically significant differences ($p < 0.05$).

[c] WSPC at pH 7.

It has been reported that the protein content of lentils was generally approximately twice fold higher than that in most cereals and similar to that in meat (Joshi et al., 2017). Boye et al. (2010) previously showed that isoelectric precipitated (IEP) (at pH 4.5) lentil proteins contain more protein than chickpea proteins. The protein contents found by these authors for IEP protein extracts of red lentils and Kabuli chickpeas were 0.78 g/g and 0.64 g/g, respectively. It was shown in another study lentil protein of freeze, spray, or vacuum-dried isolate samples varied from 0.90 to 0.92 g protein/g dry protein isolate (Joshi et al., 2011). According to Barbana et al. (2011) and Barbana and Boye (2013), the protein content of the concentrates of red lentils was found as 0.78 g/g. Very similar results were reported by Toews and Wang (2013) for the crude protein concentrates from lentil, pea, and chickpea. In this study, lentils had the highest amount of total protein as 0.88 – 0.91 g/g, followed by pea, and it was found that the lowest amount belonged to chickpea as 0.65 – 0.74 g/g. In another study conducted by Aydemir and Yemenicioğlu (2013), the protein contents of Turkish Kabuli-type chickpea extracts, red lentil cultivars extracts, and soy concentrate were determined as 0.73, 0.91, and 0.70 g/g. However, processing factors such as dehulling and grinding before protein extraction, extraction temperature and time used for extraction, centrifugation force and time, and so on can all have an impact on protein purity and yield (Russin et al., 2007). As a result, it is difficult to trace these variations in protein contents of legume seeds and differences in the process conditions could explain the changes in the reported results. Nevertheless, our results were in good agreement with previous studies on legume proteins in which protein isolates produced by alkaline extraction and isoelectric precipitation.

The WSPCs of legumes varied between 28 and 63g/100g. Chickpea, pea, and soybean did not show statistically significant differences, but WSPC of lentils had remarkable significance and the highest solubility value at neutral pH (pH 7.0) ($p<0.05$). The WSPC of lentils was almost 2-fold higher than the average WSPC of other legumes. (Table 5.1).

The ability to understand the functional properties of protein isolates or extracts is critical for their potential usage in food applications. Solubility is one of the most important functional properties of proteins because it determines the concentration of protein in a solution that is in equilibrium with protein-protein and protein-solvent interactions and most of the other functional properties depend upon it (Damodaran, 1997; McClements, 2002). Specifically, nonheme protein requires to be soluble in order to be absorbed in the intestine (Cilla et al., 2008). In the same manner, iron requires to be

soluble and stable in its ferrous form to be bioavailable for cells which is represented by solubility, namely bioaccessibility. Thus, the individual solubility profile of legume protein extracts was shown (Figure 5.1) and together with iron salt in free form were determined as a function of pH (Figure 5.2) For all protein extracts, a characteristic U-shaped solubility curve was obtained at pH conditions ranging from pH 2.0 to pH 8.0. For most of the extracts, the solubility was very low at pH 4.0, 4.5, and 5.0 ranging from 1% to 9%. This was an expected result because when pH was near the isoelectric point, the net charge on protein chains decreased, protein-protein interaction was at the maximum level (Aydemir and Yemenicioglu, 2013). The trend observed in the pea extract was slightly different from the other legume extracts. The pea extract showed higher solubility (40%) at pH 4.0, and lower solubility (1%) at pH 6.0. The solubility profiles (%) for chickpea and soybean were very similar. The trend observed for the chickpea extract was also in good agreement with the trend observed in the study conducted by Kaur and Singh (2007) and Ghribi et al. (2015). Studies conducted by other scientists have observed the solubility of protein isolates from different legumes to be lowest at pH 4-6 and highest between 8-9 (Boye et al., 2010b; Joshi et al, 2017).

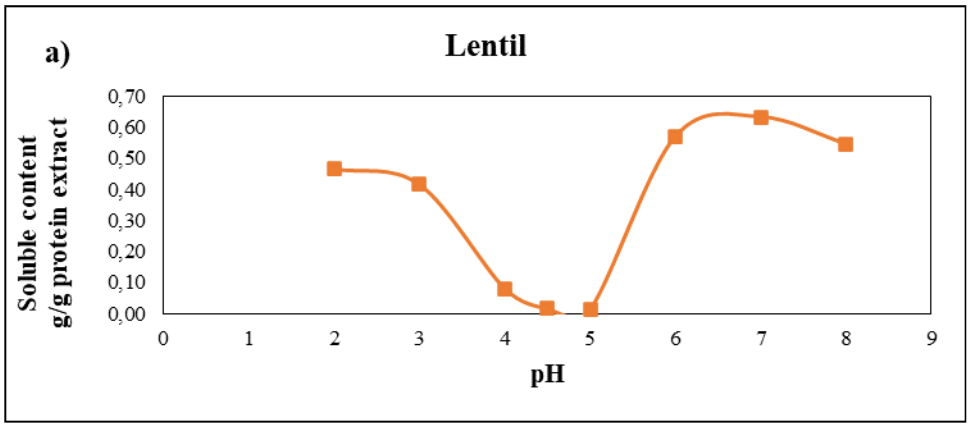


Figure 5.1. Solubility profile of (a) lentil, (b) chickpea, (c) soybean, and (d) pea as g/g protein extract.

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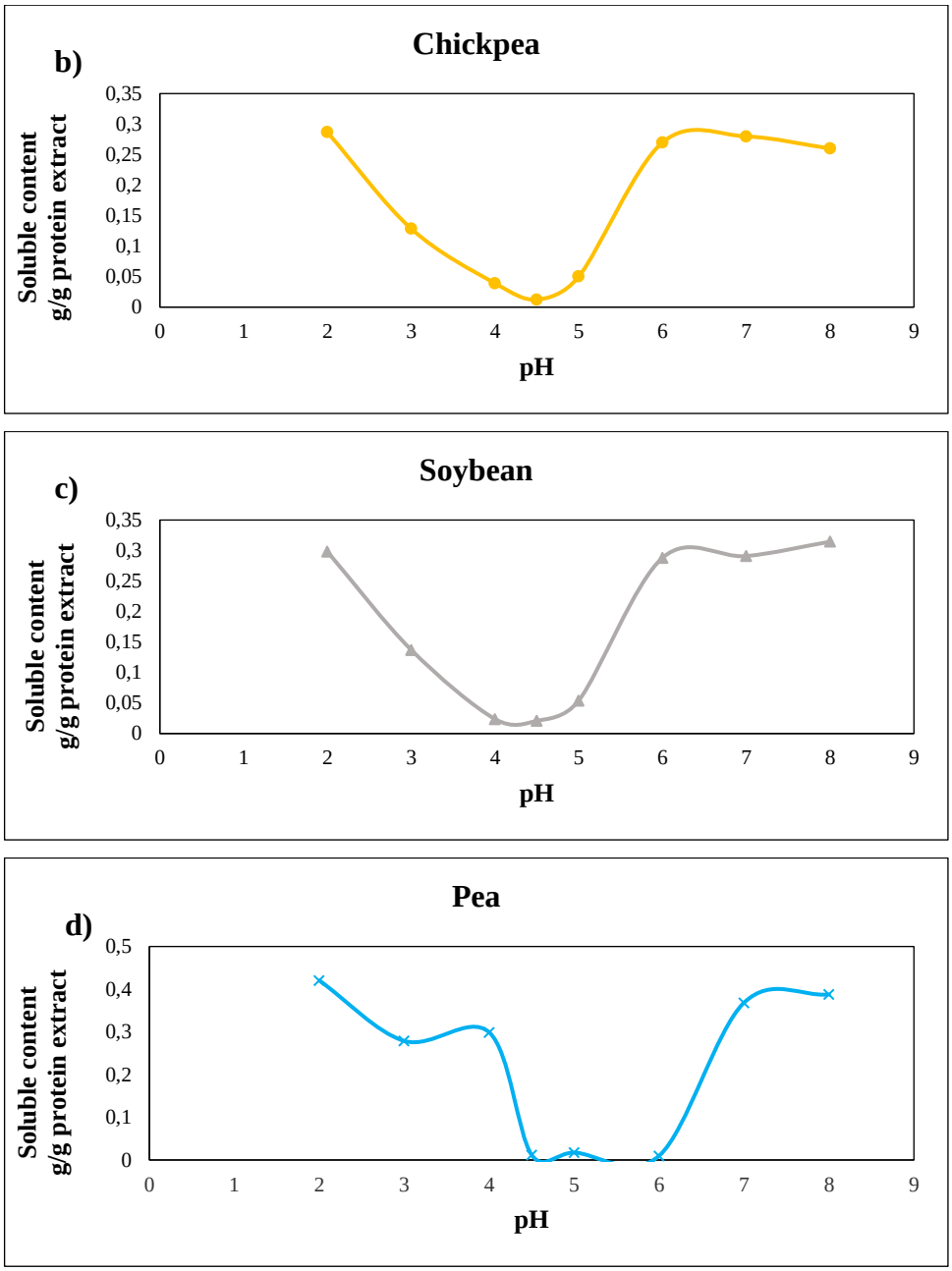


Figure 5.1 (cont.)

Both protein and iron salt showed high solubility at pH 2.0, which mimics the condition of the gastric phase of digestion (Figure 5.2). The solubility of protein was also high at the pH values ranging between pH 7-8 which mimics the condition of the intestinal phase of digestion, indicating the increasing solubility on either side of the isoelectric point. However, although the iron showed higher solubility at pH 2–5, its solubility decreased dramatically at pH values ranging between pH 3-6. In the ranges between pH 6.0 and pH 8.0 it was observed that iron was insoluble. Thus, it is clear that the acidic

pHs between 2.0 and 3.0 are highly critical since both protein and iron exist in soluble forms within this range. In contrast, at neutral and close to neutral pHs, protein is highly soluble while iron exists in an insoluble form. Furthermore, at pH 7.0 protein amino acids tend to oxidize their ionizable electron-donating groups (González-Montoya et al., 2018), and this might enhance the iron binding capacity of amino acids. This could increase iron solubility at neutral pH.

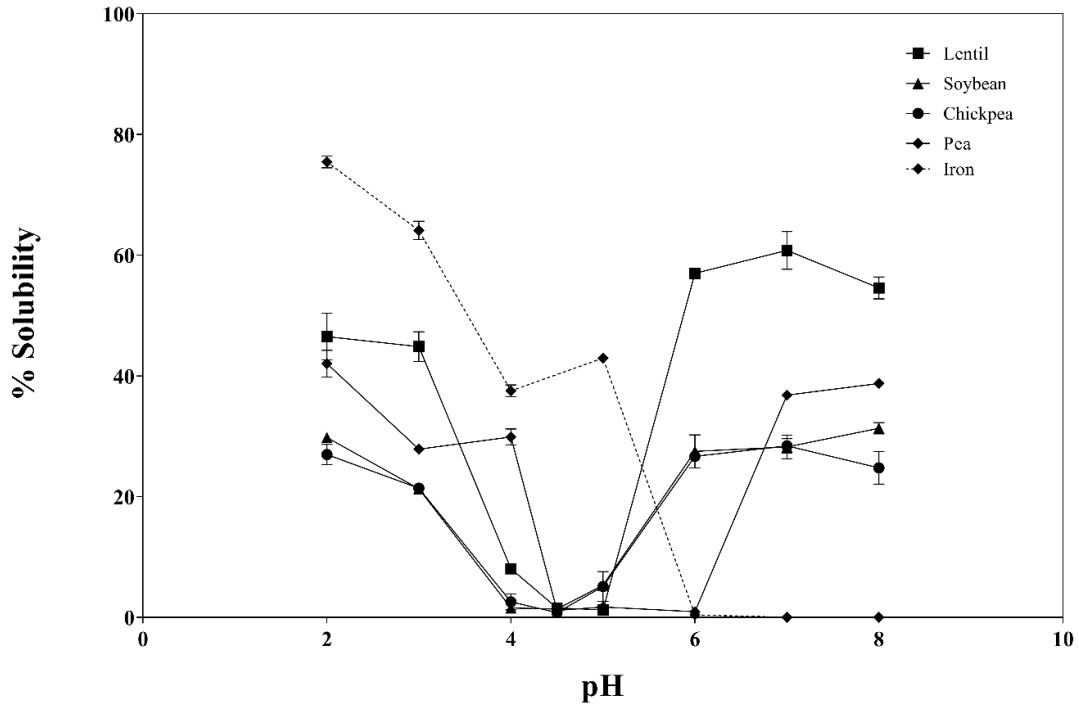


Figure 5.2. Solubility profiles of protein extracts of legumes and iron under different pH conditions

The protein functionality is mostly determined by its solubility, which is strongly influenced by pH (Pelegrine and Gasparetto, 2005). The iron salt also showed a pH-dependant solubility profile that should be known to understand its behavior in different food matrices and under different physiological conditions during gastrointestinal digestion. The iron requires to be soluble and stable in its ferrous form to remain its bioavailability for intestinal absorption (O'Louglin *et al.,* 2015, Li *et al.,* 2017). Changing the pH of the compound can influence its solubility and structural stability, which can impact iron bioaccessibility and bioavailability, respectively. The chelating agents have a protective role at this point. The protein surrounds the iron and removes the water, thereby

decreasing the formation of ferric hydroxides and increasing the solubility and bioavailability of iron. These experiments were conducted to mimic pH changes during digestion and were used to test the stability of the protein and iron compound across a wide range of physiological pH conditions along the gastrointestinal tract. Metal ions have a higher solubility at acidic pHs (3–5), which makes them easier to absorb into the intestine (Eckert et al., 2014). The iron solution was cloudy and the color of the solution was darker at pH 6-8 which also indicated the insolubility. The iron salt was insoluble at the point that the protein was soluble (at pH 7-8) suggested that chelation of iron with protein could minimize the effects of pH on iron bioaccessibility, so the bioavailability. The increase in solubility generally indicates the higher chelating level of minerals by soluble peptides (Eckert et al., 2014). Recently Eckert et al. (2014) revealed that barley protein hydrolysates and their purified fractions can remarkably increase the solubility of different metal ions. Also, Zhu et al. (2009) showed that chelated forms of iron sources (SFP and NaFeEDTA) were less affected by the change of pH than iron in $FeSO_4$.

5.7.1.2. Studies on the Determination of Fe^{2+} Chelating Capacity of Lyophilized Crude Protein Extracts

The ability of protein extracts to chelate iron minerals was tested by ferrozine assay which is the most commonly used colorimetric method for the determination of Fe^{2+} chelating capacity. In this method, ferrozine forms a colored complex with ferrous ion (Fe^{2+}-ferrozine) which can be measured spectrophotometrically at 562 nm. Ferrozine can only form a complex with free ferrous ions. Since no reaction occurs between Fe^{3+} and ferrozine the iron chelating capacity is specifically defined as the chelating capacity of Fe^{2+}. When a Fe^{2+} chelating agent is present in the environment the intensity of color decreases. Within this context, the iron chelating capacity for a peptide can be expressed as the decrease in the Fe^{2+}-ferrozine level generated compared to a control. Different studies on the chelating capacity of proteins have used EDTA as the standard. Based on this, Na-EDTA was used as a positive control in the present study.

Firstly, in order to generate a standard curve, different concentrations of Na-EDTA were prepared at two different pH conditions mimicking the gastrointestinal environment during digestion: acidic pH (pH 2.0) and physiological pH (pH 7.0), and the chelating capacities of Na-EDTA were calculated (Figure 5.3). By examining the plots

obtained as a result of the experiments indicated that at pH 7.0 the plot showed good linearity with increasing the iron chelator concentration (R^2=0,9721). On the other hand, the pH 2.0-adjusted experiment did not work very well as the experiment in the environment at pH 7. Absorbance decrease did not show exact dose-dependent linearity in the range of 0 – 50 µg Na-EDTA/reaction mixture. A linear standard curve could not be plotted properly, which means iron chelation did not occur in an efficient way as it was at physiological pH (R^2=0.8286) (Figure 5.3a).

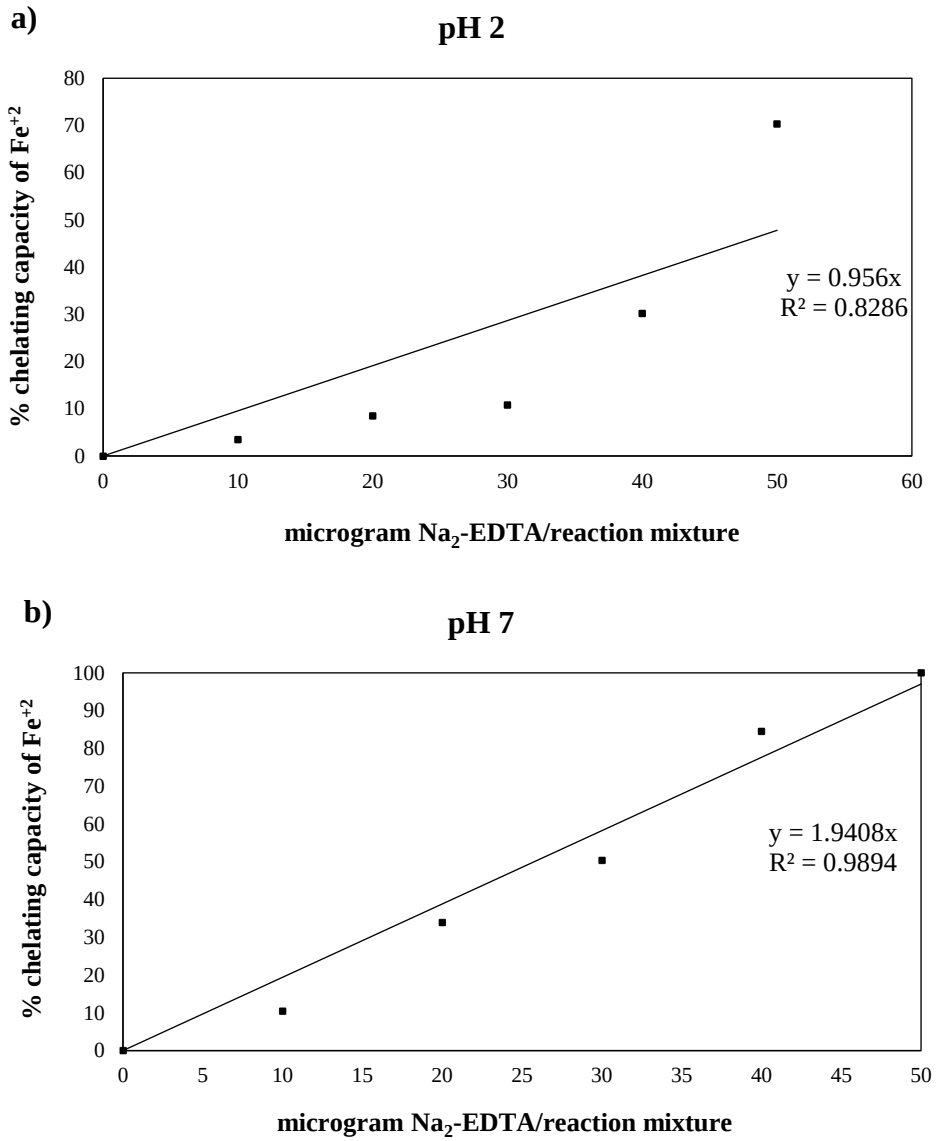


Figure 5.3. Na_2-EDTA standard curve study for determination of iron chelating capacity. (a) at acidic pH (pH 2.0), (b) at physiological pH (pH 7.0)

For further evaluation, lyophilized lentil protein extract was chosen due to its high total and soluble protein content compared to other legume extracts for initial studies in order to test the efficiency of Fe^{2+} chelating capacity assay at different pH values. At first, different concentrations of lentil protein extract were prepared and Fe^{2+}chelating capacity was checked at the physiological pH (pH 7). (Figure 5.4).

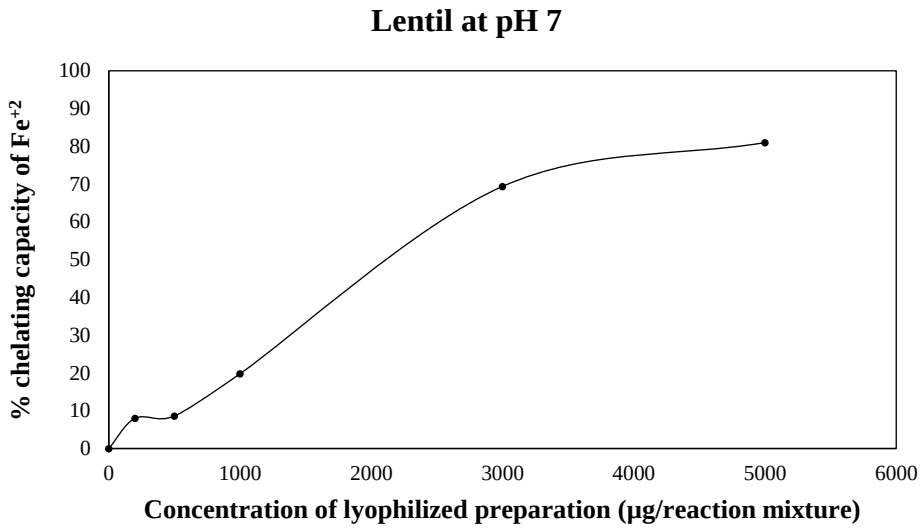


Figure 5.4. Iron chelating capacity study for lentil protein extract at physiological pH (pH 7.0).

The results indicated that the Fe^{2+}chelating capacity assay for the lentil protein extract at physiological pH worked well, in good agreement with the results obtained for the Na-EDTA Fe^{2+} chelating capacity at the same pH.

The reason for the pH 2.0-adjusted experiments did not work properly as in the experiments of physiological pH was probably the alterations in the pH. The pH of the solution may have affected the results in two ways: *(i)* it may increase the rate of Fe^{2+} autooxidation and/or *(ii)* the ferrozine used in this assay may be unstable at high acidic conditions. It has been reported that even a small variation in pH would be a large effect on the rate of Fe^{2+} autooxidation (Welch et al., 2002). And also, it has been declined by Stookey (1970) the absorbance of the iron-ferrozine complex remains stable in the pH range from 4.0 to 9.0 while the linearity and magnitude of color development are altered at acidic conditions especially less than pH 4.0. The final pH of the reaction mixture

would be another explanation for the unsatisfactory results at the acidic environment. When final pH values were checked at the end of the assay, it was seen that the values were not the same as the initial values for pH 2.0 experiments. In order to keep pH stable, one set of experiments was conducted with the buffer solutions using acetate and phosphate buffers. However, it was observed no color and the lowest absorbance values for blanks against the samples prepared with phosphate buffer. Although phosphate buffer is widely used in biological experimental systems and food applications it was revealed that phosphate ions have the ability to bond iron ions significantly (Yoshimura et al., 1992). Moreover, it was demonstrated that the rate of Fe^{2+} autoxidation was significantly affected by the buffer used in the experiments. Considering the concept of this thesis and the data obtained from these experiments, it was decided for further experiments to evaluate the iron amount of the samples at the beginning and at the end of the digestion without using any buffers, since the peptide-iron complexes that would be formed at the end of the *in vitro* digestion were important for biofunctionality.

Numerous studies documented in the literature suggest that the use of ferrous chloride solution ($FeCl_2$) as the iron source for ferrozine assay. However, to the best of our knowledge, there is no food application study with $FeCl_2$ in the literature. So, it was investigated that if an iron compound commonly used for iron fortification instead of chloride is put in this protocol the experiment will work or not by changing the chloride with ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) (Figure 5.5).

The results indicated that the Fe^{2+}chelating capacity assay for the determination of the standard curve of iron source in the form of FeSO4 at physiological pH worked well, in good agreement with the results obtained for the Na-EDTA Fe^{2+} chelating capacity at the same pH for $FeCl_2$ as an iron source.

Once using $FeSO_4$ as the iron source was proven to be effective in the construction of standard curves, ferrous ion-chelating abilities of all crude protein extracts were assessed. However, FIC was also evaluated by using $FeCl_2$ solution besides $FeSO_4$ solution to obtain comparable results (Figure 5.6). According to the data, soybean showed the highest FIC ability followed by lentil, pea, and chickpea, respectively (at the concentration of 3000 µg protein per reaction mixture). The results were in good agreement with both iron solutions used.

Considering the data obtained from both iron sources, it was decided to continue to the assay with $FeSO_4.7H_2O$ which was more convenient as fortificants in food applications. Therefore, FIC was evaluated by using $FeSO_4$ solution by narrowing the

concentration range of protein extracts (Figure 5.7). Examining the plots, lentil protein extract had the highest ferrous ion-chelating ability (FIC) at a concentration of 1500 µg/reaction mixture compared to other legumes. Approximately 73% of extracted lentil protein was soluble at the physiological pH (Figure 5.2) and it had a high chelating ability. These results were also in accordance with the literature. In a study by Aydemir and Yemenicioglu (2013), the average FIC of lentil extracts was found to be 7 to 18 times greater binding capacity than that of soy proteins. In another research, it was observed that lentil protein extracts had a higher average FIC than that of chickpea extracts (Arcan and Yemenicioglu, 2007).

These findings demonstrated that lentil proteins were more functional than the remaining chickpea, pea, and soybean proteins in terms of total protein and water-soluble protein contents, and ferrous ion-chelating ability.

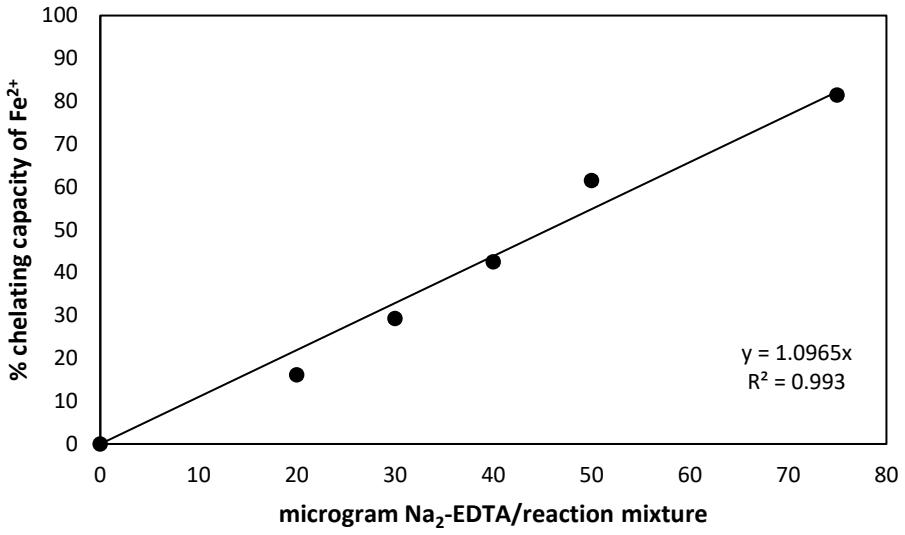
Most research to develop iron chelating peptides have focused on peptides derived from plant proteins currently. Different studies have shown the beneficial effects on iron absorption of peptides produced by enzymatic hydrolysis of legume proteins, such as soybean, lentil, chickpea, black pea (Arcan and Yemenicioğlu, 2010, Torres-Fuentes et al., 2012, Jamdar et al, 2017). Also, it was revealed that barley protein hydrolysates and their purified fractions increased the solubility of different metal ions at or near physiological pH (Eckert et al., 2014). As the pH increases to neutral or alkaline, considerably less soluble hydroxide is formed. Under the experimental conditions used for these experiments, especially above pH 5.5, the iron would probably be precipitated (Sugiarto et al., 2009). Moreover, ferrous sulfate salt is reported to be more stable in acidic environments (Spiro and Saltman, 1974).

5.8. Conclusion

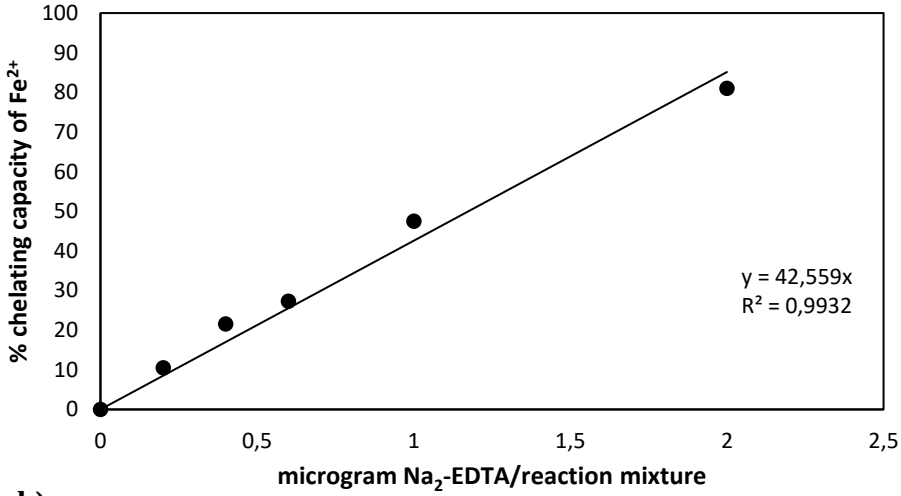
From the experiments in this chapter, it was seen that:

(i) The total protein contents of lentil, pea, chickpea, and soybean were 0.86, 0.75, 0.60, and 0.62 g/g, respectively.

(ii) For all the protein extracts, a characteristic U-shaped solubility curve was obtained. In general, the highest solubility was observed at pH 2.0 and the pH ranging



a)



b)

Figure 5.5. Na_2-EDTA standard curves for determination of ferrous ion-chelating capacity. The iron source in the form of a) $FeCl_2$, b) $FeSO_4$.

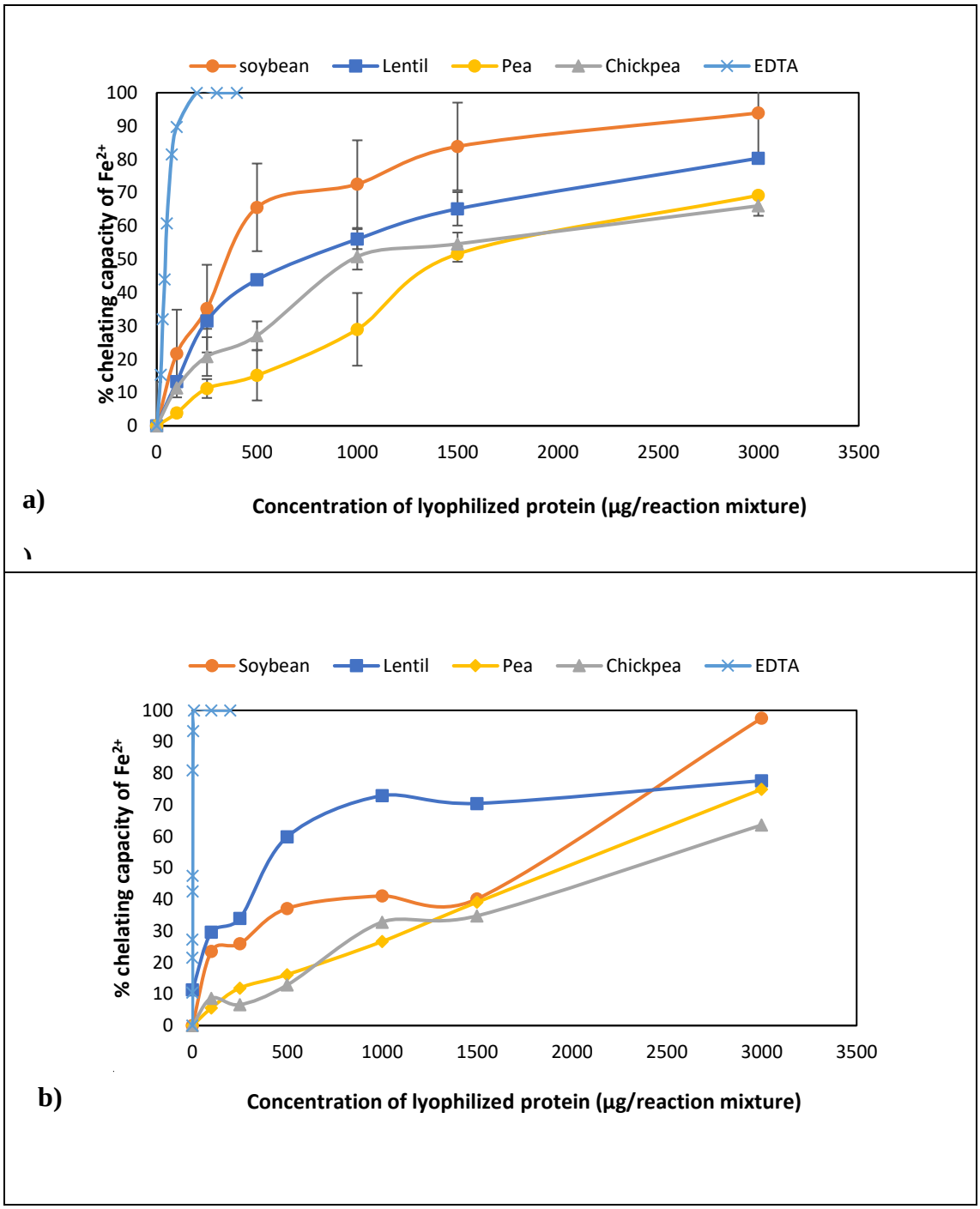


Figure 5.6. Fe^{2+} ion-chelating ability of different lyophilized crude protein extracts. The iron source in the form of a) $FeCl_2$ and b) $FeSO_4$.

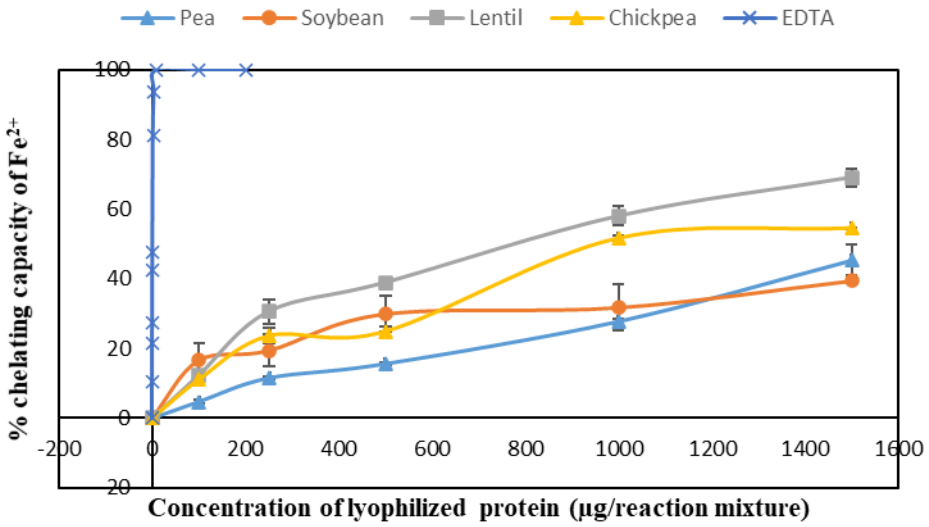


Figure 5.7. The ferrous ion-chelating capacity of lyophilized crude protein extracts of legumes.

between 7-8, indicating the increasing solubility on either side of the isoelectric point.

(iii) Lentil extract had the highest solubility at physiological pH (pH 7.0), whereas the water-soluble contents of chickpea, pea, and soybean did not show statistically significant differences ($P > 0.05$).

(iv) The results of Fe^{2+} chelating capacity indicated that the ferrozine assay for the lentil protein extract at physiological pH worked well, in good agreement with the results obtained with Fe^{2+} chelating capacity of the standard at the same pH.

(v) Ferrozine used in this assay was unstable at high acidic conditions, especially in pH values less than 4.

(vi) It was observed that phosphate was a good iron chelator, so phosphate buffer was not suitable for the experiments related to the iron binding capacity determination.

(vii) For different pH environments, ferrous sulfate as an iron source in place of ferrous chloride seemed to be suitable with this way of use considering the aim of the thesis.

(viii) Ferrous ion-chelating (FIC) abilities of all crude protein extracts were assessed with the use of ferrous chloride and ferrous sulfate solution as an iron source. For both iron solutions used, lentils showed the highest FIC ability followed by soybean

pea, and chickpea, respectively (at the concentration of 1500 µg protein per reaction mixture).

At the very beginning of the experiments, it was thought that a high free iron amount would lead to high iron bioavailability. In fact, according to recent studies chelation with protein, peptides, or amino acids increase metal solubility and so metal bioavailability. So, considering this information, our research focus has shifted from free dialyzable iron amount determination to the determination of protein-iron or peptide-iron complexes. Within this context, iron bioaccessibility was assumed to be iron solubility after *in vitro* gastrointestinal digestion.

CHAPTER 6

FORMATION OF PROTEIN-IRON COMPLEXES AND SIMULATED GASTROINTESTINAL DIGESTION

Many iron sources such as ferrous sulfate and ferrous carbonate can be used as food fortificants. However, they have low bioavailability. This may be because of several reasons. Mineral solubility is reduced by the mild alkaline environment of the intestine. Low solubility indicates a low rate of absorption and bioavailability (Eckert et al., 2016). Furthermore, iron bioavailability is limited at physiological pH in the presence of peptic digestion because ferrous ions (Fe^{2+}) are rapidly oxidized to the insoluble ferric (Fe^{3+}) form, which must be first reduced by the enzyme before being absorbed by enteric cells ((Torres-Fuentes et al., 2011, Caetano-Silva et al., 2015 and Caetano-Silva et al., 2018). As a result, substances that keep iron soluble and stable in the gastrointestinal tract by keeping it in the ferrous form boost iron bioavailability.

Peptides derived from food protein extracts or hydrolysates have recently attracted interest as novel metal chelators. When compared to inorganic salts alone, the chelated minerals are less prone to chemical interactions with the environment and are more stable (Caetano-Silva et al., 2018), with a comparable or increased effect compared to inorganic salts alone (Eckert et al., 2016). Amino acids and some other organic acids also help to improve iron absorption by balancing the pH of the intestinal contents. (Torres-Fuentes et al., 2012). The idea of using iron-protein or iron-peptide complexes could be an alternative strategy to overcome the problems related to iron fortification.

Gastrointestinal digestion has a significant impact on the biological activity of food-derived peptides, allowing the synthesis of new active fragments with improved function or, on the other hand, resulting in fragments with reduced or no activity. (Gonzales-Montoya et al., 2018). These bioactive peptides can also be yielded by previous *in vitro* protein hydrolysis (Torres-Fuentes et al., 2011). Following digestion, nutrients are absorbed from the highly polarized layer of enterocyte cells of the intestinal

epithelium, which also functions as a barrier to hinder the absorption of higher molecular weight substances (>300 Da) such as dietary proteins. Digestive enzymes convert proteins into smaller peptides and individual amino acids, which enter the systemic circulation via transcellular or paracellular channels, limiting the absorption of complete food proteins (Markell et al., 2017). Hence, legume peptide-iron complexes might be used as dietary supplements to increase mineral solubility and bioavailability. Furthermore, because these peptides have strong antioxidant properties, they might help to reduce iron oxidation in the gastrointestinal tract and/or in food storage.

The objective of this chapter was the formation of protein-iron complexes from protein extracts of legumes and to determine the optimum conditions for gastrointestinal digestion that would serve as key data before bioavailability experiments in the cell culture.

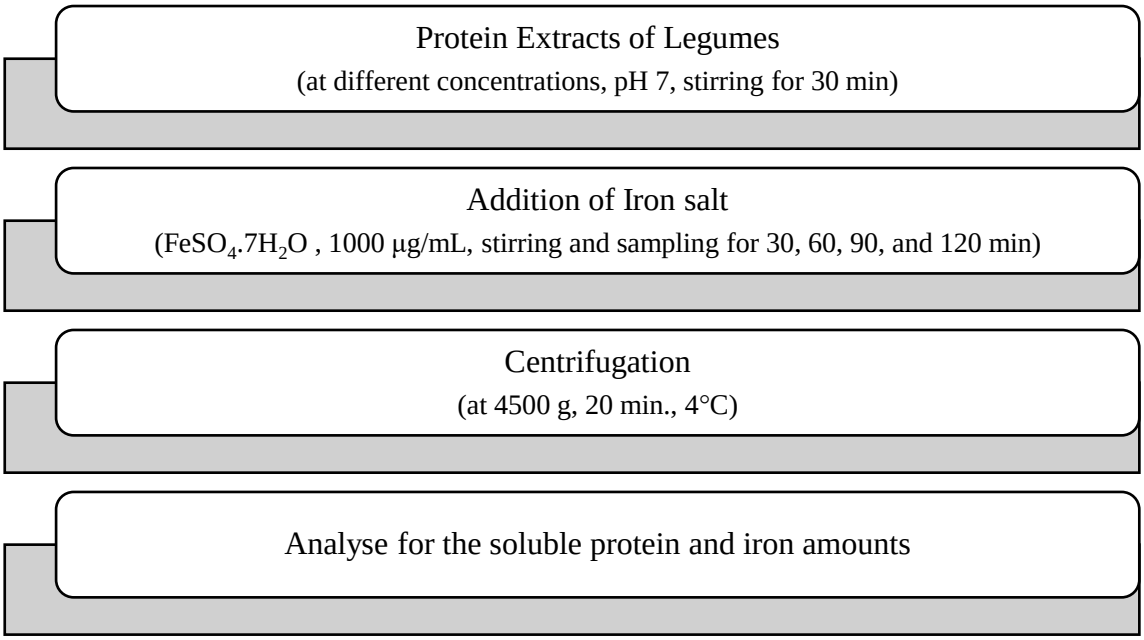
6.1. Formation of Protein-Iron Complexes

The protein extracts of all legumes were dispersed in deionized water, and the pH was adjusted to 7.0 using 0.1 mol/L NaOH. The dispersions were kept at room temperature and stirred (100 rpm) for 30 min with a magnetic stirrer. $FeSO_4.7H_2O$ as the iron source was then added into the protein solutions to reach the protein:iron ratios of 10:1 (10mg:1mg in 1mL), 20:1 (20 mg:1mg in 1mL), 40:1 (40mg:1mg in 1mL), or 60:1 (60mg:1mg in 1mL). In other words, the initial iron content was fixed as 1000 µg/mL and then the concentrations of proteins were adjusted according to the amount of iron. The soluble protein content and iron content of the solutions were then monitored and determined according to the Bradford assay and ferrozine assay described previously in Chapter 4, Section 4.4 by taking samples at 30-min intervals for 2h (Figure 6.1).

6.2. Determination of Simulated Gastrointestinal Digestion Assay for Protein Extracts

Three different *in vitro* digestion assays mostly used in the literature for legume proteins were applied to the samples to achieve high digestion efficiency. All three methods consisted of two steps to mimic the digestive process in the stomach (gastric digestion) and small intestine (intestinal digestion). All experiments were carried out in

50 mL falcon tubes each containing glass balls in a 100-rpm shaking incubator. For each assay, water was also digested in place of protein extract as a blank sample.



Protein:iron ratio (w/w)	5:1	10:1	20:1	40:1	60:1
Iron (µg/mL)	1000	1000	1000	1000	1000
Protein (mg/mL)	5	10	20	40	60

Figure 6.1. Diagram of the protein-iron complexation study.

Lentil protein extract was chosen as a model legume sample for the experiments due to its high content of total and soluble protein. As a first method, the extracts were digested according to the assay described in Gonzáles-Montoya et al. (2018) referred to in the text as Method 1 (M1). Briefly, protein extract (5% w/v) was dissolved in deionized water and then the pH was adjusted to pH 2.0 using 1 N HCl. Pepsin (4% w/w, protein basis) was added. The solution was incubated at 37ºC for 1 h with a stirring speed of 100 rpm. After 1-hour incubation, the pH of the solution was adjusted to pH 7.5 using 1 N NaOH and then pancreatin (4% w/w, protein basis) was added. The solution was incubated again at 37ºC for 2 h with the same speed of stirring. Digestion was stopped by boiling the samples in a water bath at 100ºC for 10 min. The digest was obtained by

centrifugation at 10000 g for 10 min at 4ºC. The supernatant was collected and stored at -20ºC until further analysis.

The second digestion assay (Method 2 - M2) was applied with minor modifications according to the method given in Eckert et al. (2006). 0.2 g pepsin/10 mL of protein extract (5% w/v) was prepared, and the solution was adjusted to pH 2.0 using 5.0 mol/L HCl. For the intestinal digestion, 0.05 g of pancreatin and 0.3 g of bile salts were dissolved in 25 mL of 0.1 mol/L $NaHCO_3$. 2.5 mL of pancreatin-bile salt solution was added per 10 mL of the sample after 1-hour peptic incubation and pH adjustment to 6.0 by dropwise addition of 1 mol/L $NaHCO_3$. Then pH was adjusted to pH 7.0 using 5.0 mol/L NaOH and the total volume was adjusted to 15 mL with 120 mmol/L NaCl and 5 mmol/L KCl. After 2-hour intestinal incubation, the digests were boiled at 100ºC for 4 min in a water bath to stop the enzymatic activity and then cooled in an ice bath. The supernatant was obtained by centrifugation at 10000 g for 25 min at 4ºC and then stored at -20ºC until further analysis.

The digestion assay (Method 3- M3) described in Oliviera et al. (2018) was applied to protein extracts as the third and final method. Initially, digestion fluids were prepared. 0.2 g of NaCl and 0.32 g of pepsin were dissolved in water; 7 mL of HCl 10% (v/v) was added and this solution was diluted to 100 mL with deionized water for gastric fluid. The pH was adjusted to pH 1.5. $NaHCO_3$ 3% (w/v) was prepared to use for pH adjustment. For the intestinal fluid 0.680 g of K_2HPO_4, 1.25 g bile salts, and 1.0 g pancreatin were dissolved in deionized water. 7.7 mL of 0.2 mol/L NaOH was added to this solution and then diluted to 100 mL. The final pH was adjusted to pH 6.8. For *in vitro* gastrointestinal digestion, 3.0 mL gastric fluid (pH 1.5) was added to 50 mg lyophilized lentil protein extract. The mixture was incubated at 37°C for 2 h. To adjust the pH to 6.8, 0.4 mL of $NaHCO_3$ 3% (w/v) was added. After the addition of 3.0 mL of intestinal fluid, the extract solution was incubated using the same conditions given in the gastric digestion step. The samples were placed into an ice bath to stop the enzyme activity, and finally, the digests were centrifuged at 10000 g for 10 min at 4ºC. The supernatant was collected and stored at -20ºC until further analysis.

6.2.1. Optimization of Simulated Gastrointestinal Digestion Assay

A Box-Behnken design was generated and conducted with three factors; which were pepsin concentration (X_1) and pancreatin concentration (X_2) ranging for each sample between (4-10% w/w protein basis) and duration of gastric digestion (X_3) ranging between 1 or 2 h. The response was determined as the soluble protein content of samples which was expressed as g soluble protein per g of protein extract (mg/g). A total of 17 experiments was conducted with 5 center points for optimization experiments.

Based on the results of the optimization study, digestion experiments continued according to the assay described in Gonzáles-Montoya et al. (2018) with minor modifications. Briefly, the pH of the protein-iron complexes that formed at the specified concentrations with the given procedure described in Section 5.1 was maintained at pH 2.0 using 1 N HCl and then digested with pepsin (8% w/w, protein basis) at 37ºC for 2 h. After 2-hour incubation, the pH of the solution was adjusted to pH 7.5 using 1 N NaOH and further digested with pancreatin (4% w/w, protein basis) at 37ºC for 2 h. Digestion was stopped in an ice bath at for 10 min. The digest was centrifuged at 4500 g for 20 min at 4ºC and then the supernatants were collected. Their free ferrous iron content and soluble protein content were analyzed immediately for further cell experiments.

6.3. Enzymatic Hydrolysis Assay for Protein Extracts of Legumes

The protein extracts of legumes (5% protein solution; w/v) were hydrolysed by the pancreatin (8% w/w, protein basis) at pH 7.5 and 37ºC for 8 hours, sampling at every hour. The resultant solution was cooled in an ice bath for 10 min to inactive the pancreatin. The supernatant was collected by centrifugation at 4500 g for 20 min at 4 °C and analysed for their soluble protein content and iron content.

6.4. Statistical Analysis

Data were statistically evaluated by one-way analysis of variance (ANOVA) using the PRISM software, version 3.02 (Graph Pad Software, Inc., San Diego, CA, USA). Significant differences between means were determined by Tukey's Multiple Comparison Test procedure at the 5% significance level. Design Expert Version 11 was

used for the statistical experimental design for all the simulated gastrointestinal digestion experiments with the response as soluble protein content (g soluble protein/g of protein extract). The results were considered statistically significant for *p* values less than 0.05.

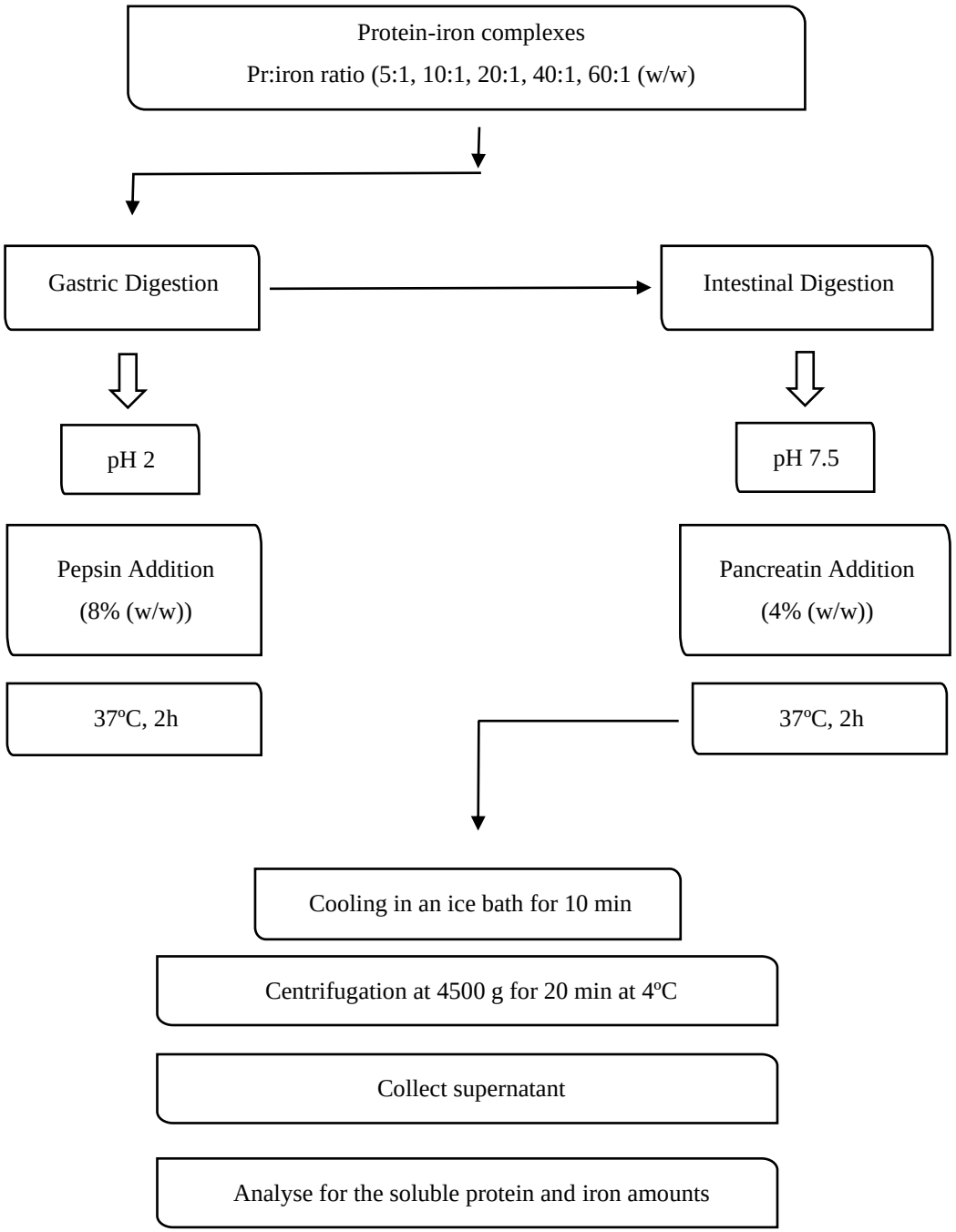


Figure 6.2. Flow diagram of the optimized and validated *in vitro* digestion conditions of the legume derived protein-iron complexes.

6.5. Results and Discussions

6.5.1. Protein and Iron Binding Profiles of Complexes at Physiological pH

The protein extracts of legumes were dispersed in deionized water, and kept at room temperature with stirring (100 rpm) for 30 min. The $FeSO_4.7H_2O$ were then added into the protein solutions to reach the specified concentrations. The iron binding profiles of legume proteins were obtained by monitoring the soluble protein contents and iron contents of the solutions at physiological pH (pH 7.0) during 2h incubation period at half-hour intervals (Figure 6.3).

The protein-iron complexes were formed at pH 7.0 because it was stated that partial deprotonation of the ionizable electron-donating groups on amino acid side chains favor coordinated iron binding (Caetano-Silva et al., 2015). The formation of complexes was completed according to the procedure given in Section 5.1 with different protein:iron ratios (w/w) including 5:1, 10:1, 20:1, 40:1, and 60:1. The protein amount in the complexes was gradually increased while the amount of iron was kept constant to be able to understand the effect of the protein content on iron chelation. After 30 min-interaction, protein solubility was decreased significantly in the complexes prepared at protein:iron ratios of 5:1, 10:1 and, 20:1 while the complexes prepared at protein:iron ratios of 40:1 and 60:1 maintained their high soluble protein content during 2h incubation period for all legume extracts (Figure 6.3A). The sharp decrease in solubility for the complexes that contained low protein:iron ratios might be explained by the reduced protein solubility or reduced detectability by the Bradford reagent due to protein aggregation mediated by extensive intermolecular binding of iron among protein molecules. Similarly, the intramolecular binding of iron molecules to reactive amino acid side chains of individual protein molecules could also reduce detectability by the Bradford reagent. Therefore, the Lowry assay was used as an alternative method to confirm Bradford assay data to be able to understand if the decrease in solubility derived from the method used or not. Very similar results were observed (data not shown) supporting previous results.

As a reverse approach, free iron levels were measured in the samples of protein–iron complexes, and iron profiles during the formation of complexes could also be seen

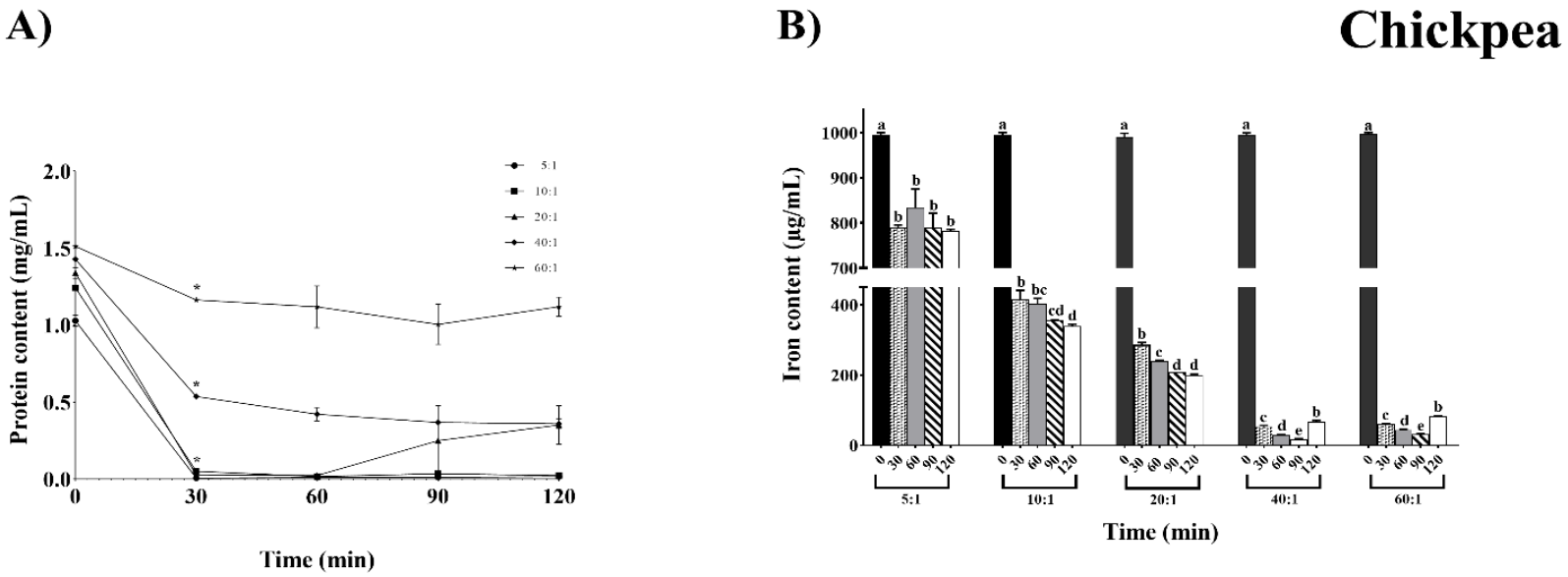


Figure 6.3. Iron binding profiles of legume proteins at different ratios under physiological pH conditions with respect to time. (A) Protein content and (B) iron content. The amounts are shown as protein:iron ratio (5:1 (5 mg:10 mg in 1 mL), 10:1 (10 mg:1 mg in 1 mL), 20:1 (20 mg:1 mg in 1 mL), 40:1 (40 mg:1 mg in 1 mL), and 60:1 (60 mg:1 mg in 1 mL)). Initial load of iron was 1000 µg/mL. Bars with lowercase letters are significantly different ($p < 0.05$). Results are shown as mean ± standard deviation.

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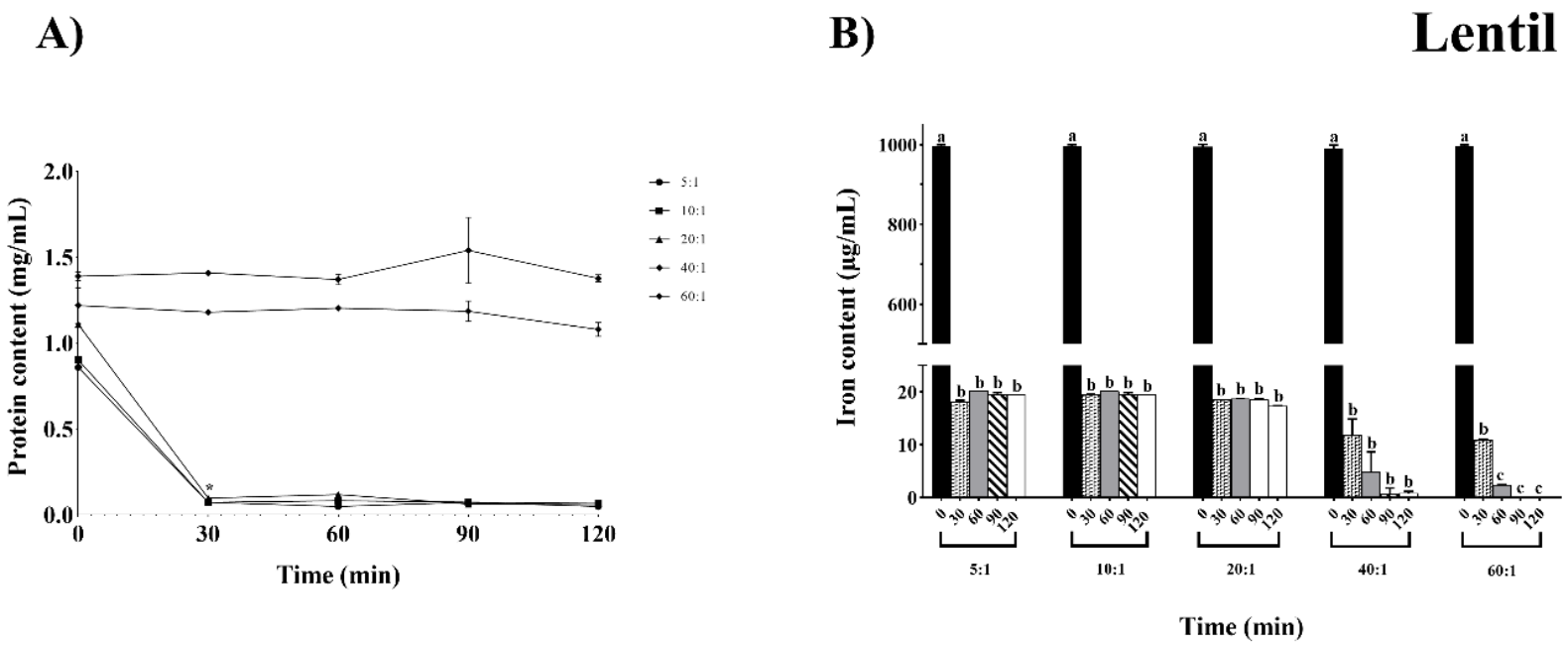

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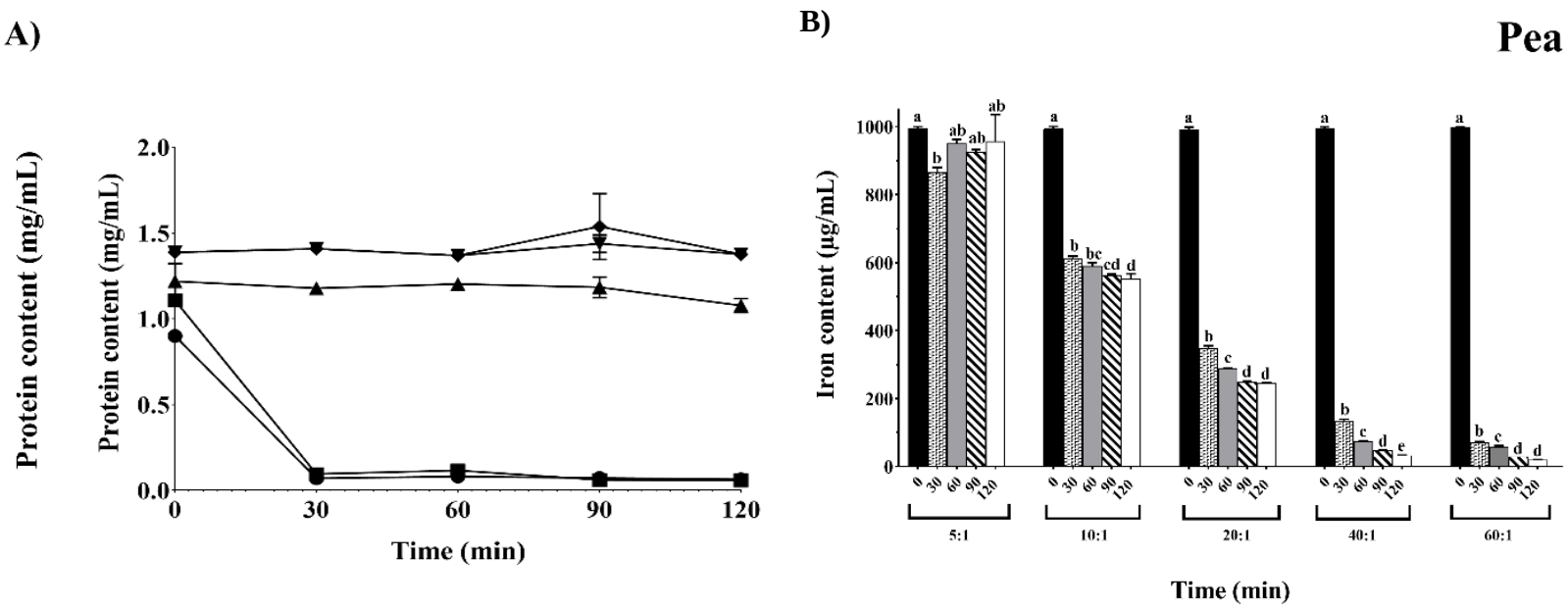

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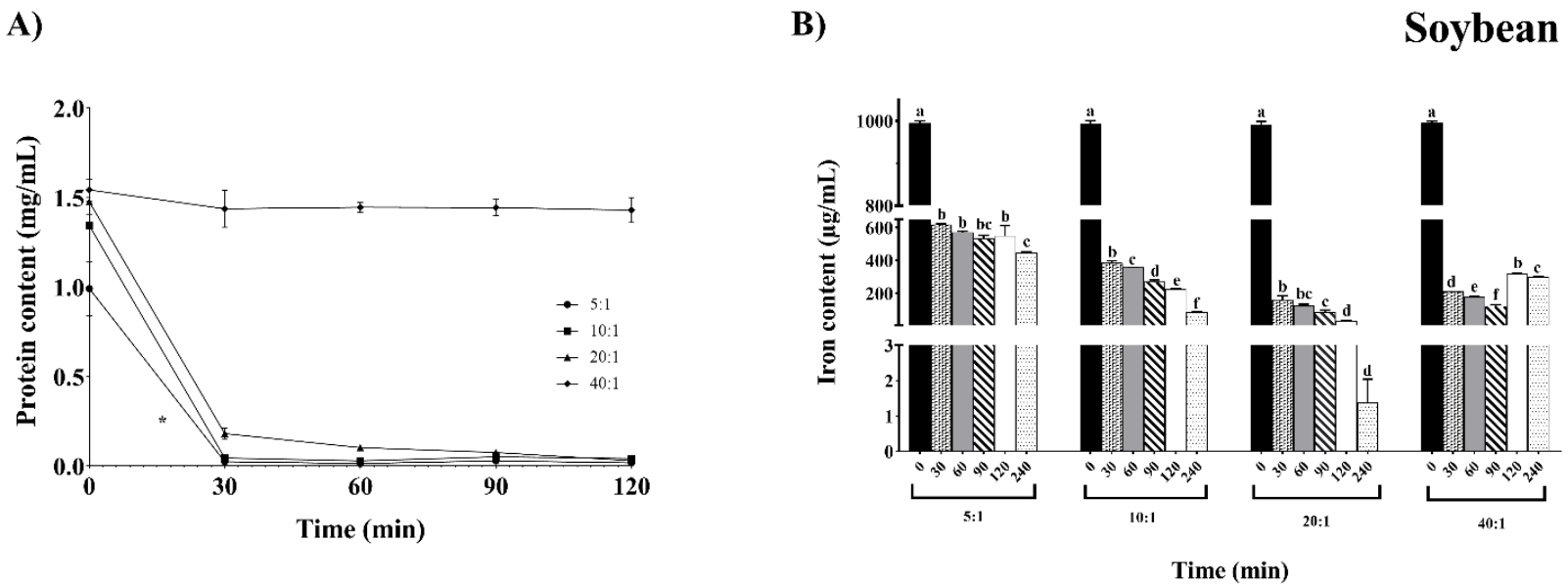


Figure 6.3. (cont.)

in Figure 6.3B. As can be seen from the figure above, the 30-min incubation period for lentil protein-iron complexes caused chelation of a minimum of 98% of the total free iron (from 1000 μg/mL to 20 μg/mL) at all ratios. A residual free iron (1.8 to 2%) was maintained in the samples that contained protein-iron complexes at protein:iron ratios of 10:1 and 20:1 during 2h incubation period. However, iron binding and chelation occurred very extensively in the samples of protein-iron complexes at protein:iron ratios of 40:1 and 60:1, thus, free soluble iron content in these solutions was reduced to undetectable levels after 90 min of interaction. In contrast to lentils, the situation was different for chickpea protein extracts. It was observed that the incubation of protein-iron complexes for 30-min caused chelation of only 20% of the total free iron at a ratio of 5:1 and, at the end of the 2h-period, this chelation ratio remained almost the same as in the first 30 min-incubation. For higher ratios of complexes (40:1 and 60:1), iron binding and chelation occurred very extensively approximately 94% of chelation after 30-min interaction. However, it was observed some instability problems when incubation continued. The free soluble iron content was almost 13 times higher at the 120-min time point compared to the amount at a ratio of 60:1 for 30-min interaction and the same instability problem occurred at the complexes with higher ratios of soybean. It was observed a slight increment in the free iron amount at the end of the incubation. Finally, complexes that contained pea protein showed a similar trend with complexes that contained lentil protein. After 30-min interaction, the iron content remained almost the same as that of low concentration complexes, whereas iron binding and chelation occurred very extensively in the protein-iron complexes that had high concentrations of protein. These results clearly showed that much of the iron binding by protein chelating groups was completed within 30-min interaction at a wide protein:iron ratios range and iron binding capacity of the protein was correlated with an increased amount of protein. Therefore, it was decided to keep formation time as 30 min order to prevent iron oxidation and precipitation.

The overall binding profiles obtained from protein-iron complexes suggested that in the complexes consisting of high protein amount might suffer instability if the formation time kept longer. Also, it was seen that the protein-iron complexes with high ratios were soluble at pH 7.0 while complexes with low protein:iron ratios might suffer from solubility and aggregation formation. However, the chelating agents could provide an advantage as a protective role by hiding the iron, and as a consequence, this might increase the bioavailability of iron. The different solubility features of proteins (high solubility) and iron (low solubility) at physiological pH could be used to increase the

solubility of iron after the formation of protein-iron complexes. It was thought that the degree of solubility and aggregation (the degree of polymerization) of a chelating agent (protein)-iron complexes should play a significant role in iron bioavailability as physiological pH conditions change along the gastrointestinal tract, in other words during gastrointestinal digestion. Considering the data obtained from the solubility experiments, it can be said that the iron salt was insoluble at the point that the protein was soluble (at pH 6-8), suggesting that chelation of iron with protein could minimize the adverse effects of pH on iron bioaccessibility and bioavailability. Eckert *et al*. (2014) revealed that barley protein hydrolysates and their purified fractions provided higher solubility of different metal ions unmistakably and concluded that higher solubility of metal ions at acidic pH values (3–5) facilitated their bioaccessibility through higher absorption.

6.5.2. Simulated Gastrointestinal Digestion Assay for Protein Extracts

Three different simulated gastrointestinal digestion assays were applied to find out the convenient method for the samples used for this thesis. For this purpose, lentil protein was chosen as modal for digestion experiments due to its high content of total and water-soluble protein, as stated previously. Digestion efficiency was determined based on the increase of soluble protein content of lentil proteins and after digestion, their Fe^{2+} ion-chelating ability was evaluated. As seen in Figure 6.4a Method 2 (M2) used for digestion gave the highest efficiency based on the soluble protein content, approximately 5-fold, and 6-fold, compared to other methods Method 1 (M1) and Method 3 (M3), respectively. This may be due to differences in pepsin concentration and the presence of bile salt in the intestinal phase. However, when Fe^{2+} ion-chelating abilities were examined by looking at the blanks after digestion it was observed that Method 2 caused peptides with considerably high chelation ability (Figure 6.4b). This finding might be related to the binding ability of gastrointestinal fluids, possibly there were strong interactions between iron and the available functional groups on digestive fluids and enzymes (Caetano-Silva et al.,2018). Moreover, it was shown that bile salt has a strong ferrous ion chelating ability (O'Loughlin et al., 2015). Therefore, it can be concluded that it was not appropriate to use bile salt in the experiments due to the results in falsely increased iron chelation. For further experiments, it was decided to continue with the M1 method with its lack of interference with iron ions.

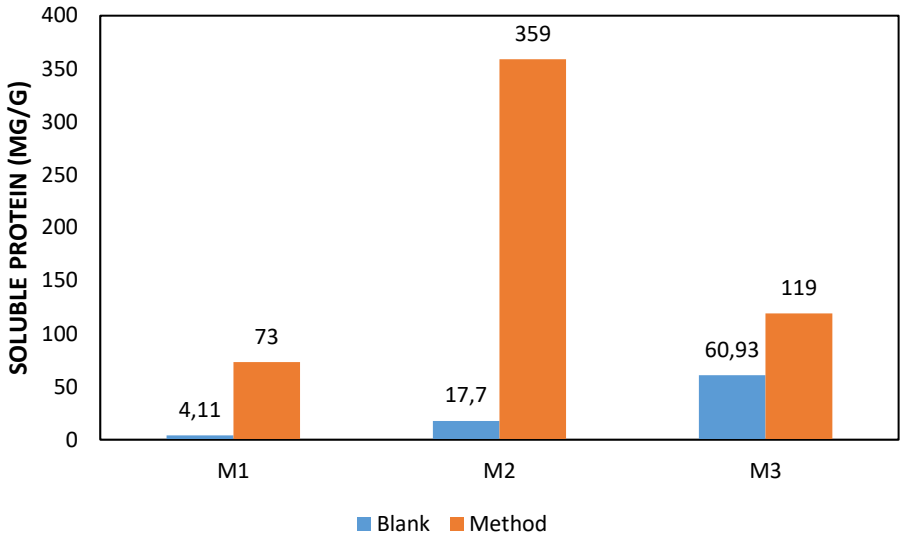
In the digestion protocols applied, the methods other than the third one (M3) ended up boiling. Since the main purpose of the studies in this thesis is to form edible protein - iron complexes and to model the human digestive system, boiling would not be suitable for the main purpose. Hence, in the first method which was the most suitable method for the samples considering the main purpose of the thesis, digestive enzyme inactivation was supplied via ice bath in the place of boiling (Figure 6.5). This additional experimental set was allowed us to make comparisons about the effects of digestion termination on protein-iron complexes for further experiments.

As can be seen from the figure, a statistical difference was found between boiling and cooling in an ice bath for stopping the enzyme activation. The boiling step at the end of the digestion caused a decrease in the soluble protein content of the legume compared to cooling with ice suggesting that a possible decrease in the binding sites for iron. As mentioned previously, boiling was a method that did already not fit the main purpose of the thesis, even if it did, it still would not be suitable since it had a negative effect on soluble protein content, quite likely due to the denaturation of proteins. In consequence, it was decided to stop the enzyme activity by cooling in an ice bath for digestion experiments.

6.5.2.1. Optimization of Simulated Gastrointestinal Digestion

Optimization of simulated gastrointestinal digestion was performed according to the Box-Behnken experimental design given with details in Section 6.3. According to the literature, concentration of digestion enzymes and duration of gastric phase were chosen as important factors for effective digestion. The actual levels of these variables and the response variable were tabulated in Table 5.1. Based on the results, the maximum soluble protein amount (92.4 mg/g) was obtained at 7% and 4% of pepsin and pancreatin concentration, respectively with 2-hour gastric digestion.

a)



b)

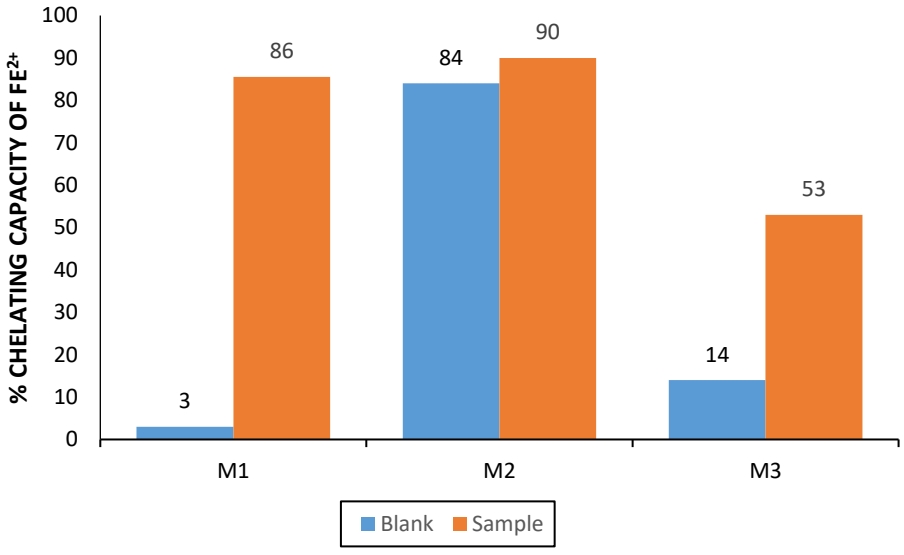


Figure 6.4. **a)** Soluble protein content and, **b)** Fe^{2+} ion-chelating ability (FIC) of lyophilized lentil protein extract and water as blank after subjecting to different *in vitro* digestion assays. FIC was expressed as a percentage (%). M1: Method 1; M2: Method 2; M3: Method 3.

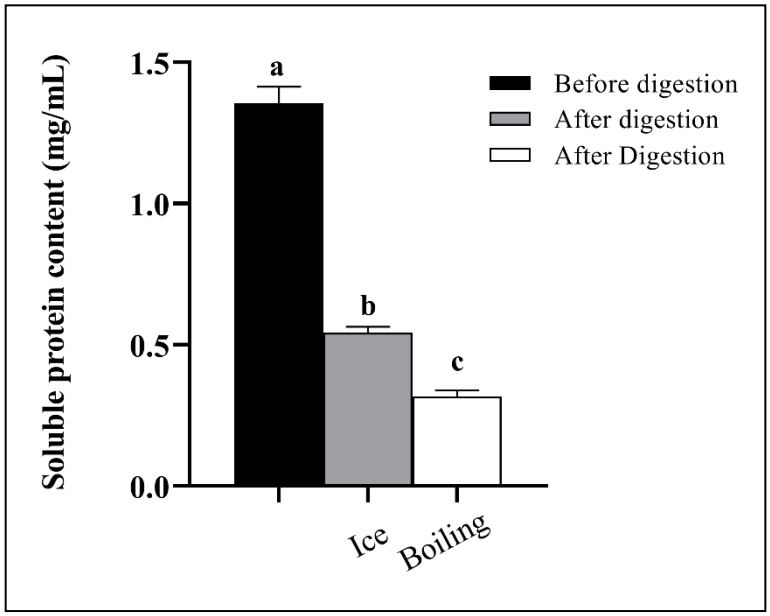


Figure 6.5. Effect of termination method on the soluble protein content of samples for *in vitro* gastrointestinal experiments.

The optimization results were discussed below according to the results of the ANOVA presented in Table 6.2. Since there were some insignificant terms, the full model was reduced by eliminating these terms and re-evaluated.

The p-value of the model was 0.0003 which indicated that the constructed model was significant ($p<0.01$) and P-values less than 0.05 indicated model terms were significant although the results obtained from the experiments seemed close to each other, they were significantly different statistically. In this case, X_3, X_1X_2, X_1X_3, X_1^2, X_2^2, X_3^2, $X_1^2X_2$, $X_1^2X_3$, namely duration of gastric digestion and the interactions between enzyme concentrations and between pepsin concentration and duration were significant model terms. According to p-values, although concentrations of pepsin (X_1) and pancreatin (X_2) were not significant factors whereas some of their interactions were significant, they remained in the model because of the hierarchy principle. The Lack of Fit F-value of 0.76 implies the Lack of Fit is not significant relative to the pure error. Non-significant lack of fit is good because it is desired for the model to fit. The Predicted R^2 of 0.7196 is not as close to the Adjusted R^2 of 0.9448 as one might normally expect. Adeq Precision measures the signal to noise ratio. A ratio greater than equation that expressed the degree of hydrolysis given as soluble protein in terms of coded factors was as following.

Table 6.1. Box-Behnken design used in the optimization of gastrointestinal digestion assay with respect to the degree of hydrolysis (DH) expressed as soluble protein amount (mg soluble protein per g of protein extract).

	Actual level of variables			**Response variable**
Run no	**Pepsin Concent. (%)**	**Pancreatin Concent. (%)**	**Duration of gastric digestion (h)**	**Soluble protein amount (mg/g)**
1	7	4	1	68.7
2	7	7	1.5	71.2
3	10	10	1.5	77.0
4	10	7	1	74.8
5	7	10	1	67.1
6	4	7	2	82.7
7	7	7	1.5	73.5
8	10	7	2	88.5
9	4	10	1.5	85.6
10	7	10	2	87.9
11	7	4	2	92.4
12	10	4	1.5	78.8
13	7	7	1.5	73.9
14	4	7	1	82.1
15	7	7	1.5	70.0
16	4	4	1.5	76.8
17	7	7	1.5	70.1

Table 6.2. Analysis of variance (ANOVA) for reduced cubic model to determine optimum conditions for simulated gastrointestinal digestion assay.

Source	Sum of Squares	df	Mean Square	F value	p-value	
Model	921.8	10	92.13	28.40	0.0003	*Significant*
X_1	8.20	1	8.20	2.53	0.1629	
X_2	9.30	1	9.30	2.87	0.1413	
X_3	495.6	1	495.6	152.63	< 0.0001	
X12	28.09	1	28.09	8.66	0.0259	
X13	42.90	1	42.90	13.23	0.0109	
X_1^2	123.5	1	123.5	38.06	0.0008	
$X2^2$	24.56	1	24.56	7.57	0.0332	
$X3^2$	100.6	1	100.6	31.04	0.0014	
X_1^2X2	21.45	1	21.45	6.61	0.0422	
$X1^2X3$	114.0	1	114.0	35.15	0.0010	
Residual	0.1946	6	3.24			
Lack of Fit	0.0535	2	2.68	0.7590	0.5255	*not significant*
Pure Error	0.1411	4	3.53			
Cor Total	940.73	16				
Std. Dev.	1.80			R-Squared	0.9793	
Mean	77.71			Adj R-Squared	0.9448	
C.V. %	2.32			Pred R-Squared	0.7196	
				Adeq Precision	17.464	

Degree of hydrolysis (expressed as g soluble protein/g protein) = y = + 71.72 – $1.01X_1$ -1,53*X_2 + 11.13*X_3 – 2.65*X_1X_2 + 3.28*X_1X_3 + 5.41*X_1^2 + 2.42*X_2^2 + 4.89*X_3^2 +3.28*$X_1^2X_2$ – 7.55* $X_1^2X_3$ (5.1.)

To optimize and fix the conditions, a variety of graphs were examined (Figure 6.6). It was observed that a high amount of pepsin (Figure 6.6a) and a low amount of pancreatin (Figure 6.6b) with 2-hour incubation in the gastric phase (Figure 6.6c) led to effective digestion. 3D response surface plots (Figure 5.6d and 5.6e) were also supported this finding.

To fix the digestion parameters, numerical optimization was applied with maximizing the response (Figure 6.7). Ramps graphs (Figure 6.7b) presented a good visual of the best factor settings with the greatest overall desirability of the predicted response. Analyses and graphs predicted higher digestion efficiency with high desirability if the conditions were set as 8% pepsin concentration, 4% pancreatin concentration with 2-h gastric incubation (Figure 6.7c). These parameters were experimentally validated and different protein:iron were utilized in digestion regarding the validated protocol. This information allowed us to figure out the optimal digestion conditions for protein-iron complexes. The selection of digestion protocol was critical for *in vitro* cell culture experiments to minimize the cofounding factors including minerals, bile acids, etc. It was only intended to digest protein-iron complexes by pepsin and pancreatin, which is a very common method for protein digestion.

6.5.3 Simulated *in vitro* Digestion Studies of Protein-iron Complexes

Effects of various digestive enzymes and strong pH fluctuations were the challenging conditions of the gastrointestinal environment and protein-iron complexes must survive from these challenges to exhibit a beneficial effect (Lafarga and Hayes, 2017). Solubility and stability are the critical factors for mineral bioavailability. Protein-iron complexes that will be formed should be stable in the gastrointestinal tract in order to maximize the absorption through the intestines. Trace minerals are protected from the harsh gastrointestinal environment and chemical reactions during digestion in favor of their electrical neutrality which provides stability to chelates. Inorganic mineral compounds are found typically in oxide or sulfide form and with the stomach's low pH they are released and ionized which means that reactivity with other products. because the electrically charged forms of the minerals are able to react with other products generated during digestion (CFNP TAP Review, 2002). According to the literature, it was stated that the consecutive digestion system causes the formation of a greater variety of peptide sequences with different bioactivities. They can act alone or synergistically. Because using two digestive enzymes with different active sites during digestion means more cut-off sites, so that increased exposure of amino and carboxyl-terminal aromatic amino acid

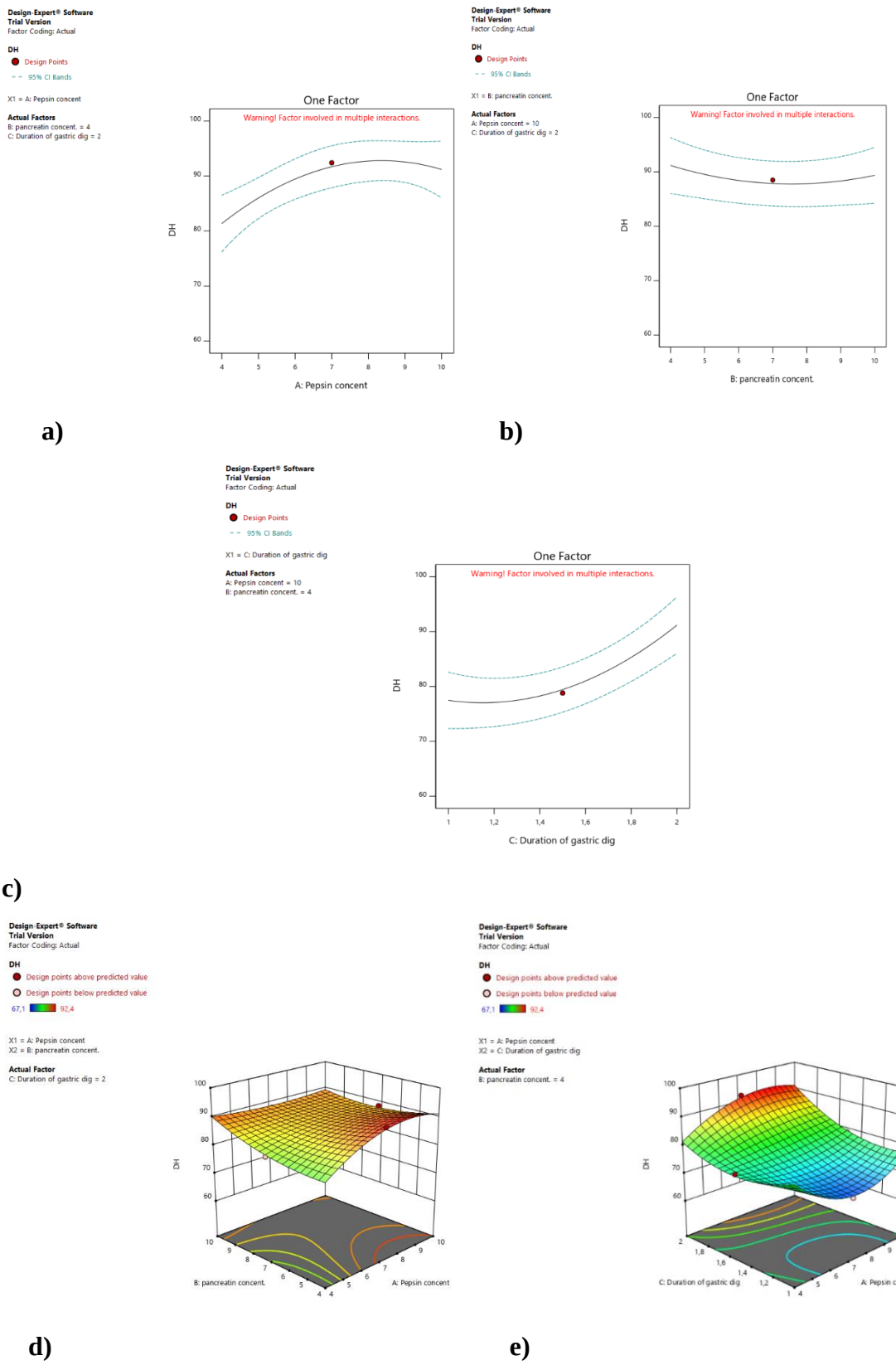
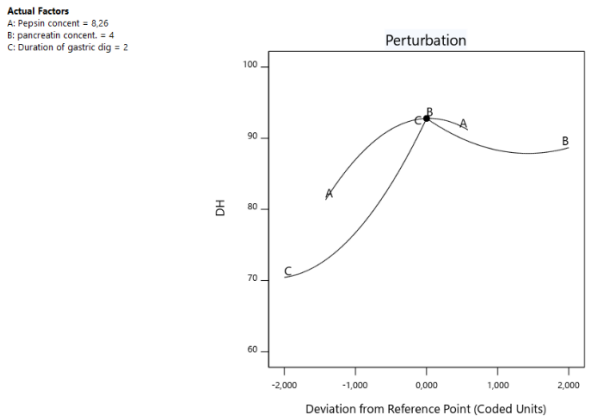


Figure 6.6. One factor plots of a) pepsin concentration, b) pancreatin concentration, c) duration of gastric digestion. d) and e) Response surface plots showing factors and their interactions.

a)



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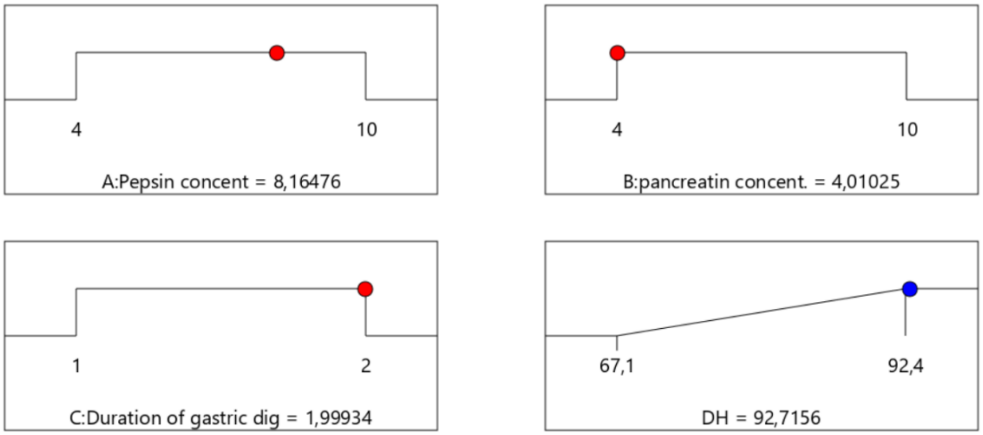

c)

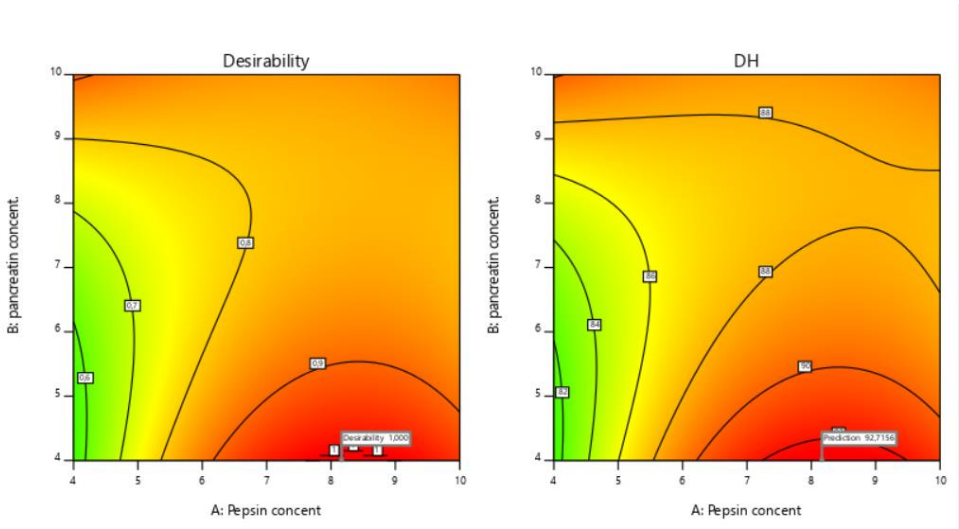

Figure 6.7. a) Perturbation plot showing the interaction between enzyme concentrations and duration of gastric phase; b) Numerical optimization; c) Desirability plots.

residues meaning that more metal binding sites. (Polanco-Lugo et al., 2014, Sanchez-Chino et al., 2018).

Protein and iron interactions occur through the binding sites of amino acids of peptides. Then another question was how protein and iron complexes behaved during digestion. The hydrolyzation of protein-iron complexes leads to the production of peptide-iron complexes, which are the functional form of protein-iron complexes. In line with this information, the stability of protein-iron complexes was evaluated under simulated *in vitro* digestion conditions. These experiments were based on the following points (Caetano et al., 2015): if the protein-iron interaction remains stable at gastric pH (pH 2) and/or the activities of digestive enzymes induce the breaking of the complexes, or if the iron released during digestion will precipitate when the pH is adjusted to intestinal pH. Considering the opposite case, if the complexes remain stable against gastric digestion conditions, whether the iron will remain soluble or not at the end of the digestion at the intestinal pH (pH 7.0). Thus, free iron remained in the solution was determined after simulating digestion to evaluate the stability of protein-iron complexes.

Initially, protein-iron complexes were formed. After that simulated *in vitro* digestion studies were carried out with different protein:iron ratios (w/w): 5:1, 10:1, 20:1, 40:1, and 60:1, respectively (Figure 5.8) under optimized and validated digestion conditions.

It was observed that free iron was not detected in the complexes that contained protein:iron ratios of 10:1 to 60:1 at the end of the digestion for lentils (Figure 6.8A). For these experiments, the 5:1 ratio was also included to be able to test whether all iron was bound to protein in any ratio lower than 10:1. After digestion, unbound free iron was observed in the sample at a ratio of 5:1 indicating that protein amount was critical for chelation of iron. It was significantly important that the digest did not contain free iron in terms of downstream cell experiments to distinguish the functionality, if there was, that came from free, or peptide-bound iron. It was observed that protein:iron complexes at ratios of 20:1, 40:1, and 60:1 did not also have free unbound iron minerals. However, the 10:1 ratio was selected for the cell culture experiments since it was the first ratio that iron was not been detected in the solution after digestion. It was thought that minimizing the hydrolyzed protein amount at the minimum level could provide a beneficial effect for the functionality. In other words, digested protein-derived peptides might also affect cellular

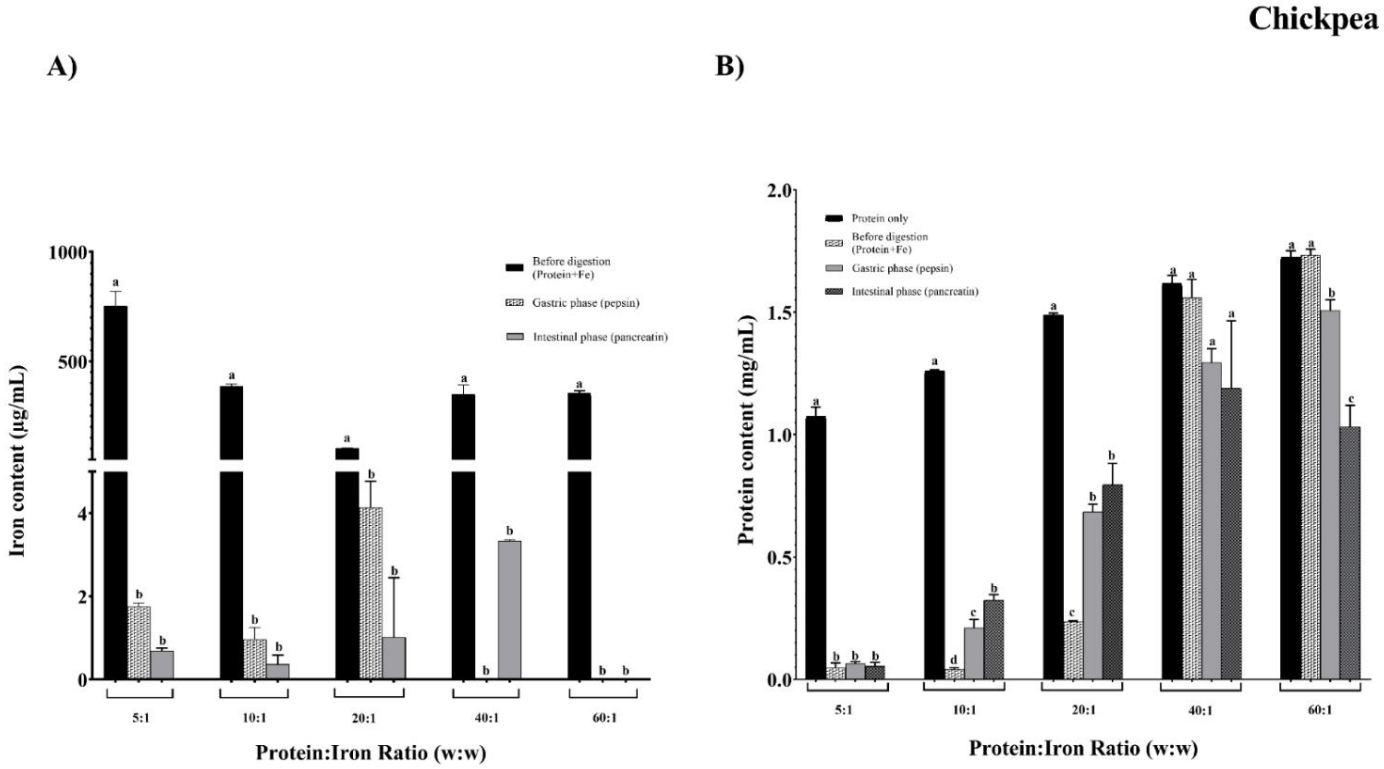

Figure 6.8. The profiles of (A) bioaccessible free iron content (B) soluble protein content of protein–iron complexes derived from legumes at different protein:iron ratios (w/w) after *in vitro* digestion. Protein–iron was combined in the solution following pepsin incubation. Next, this hydrolysate was incubated with pancreatin. Protein and iron contents were measured before hydrolysis, after pepsin incubation, and then pancreatin addition. Bars with different lowercase letters are significantly different ($p < 0.05$). Results are shown as mean ± standard deviation.

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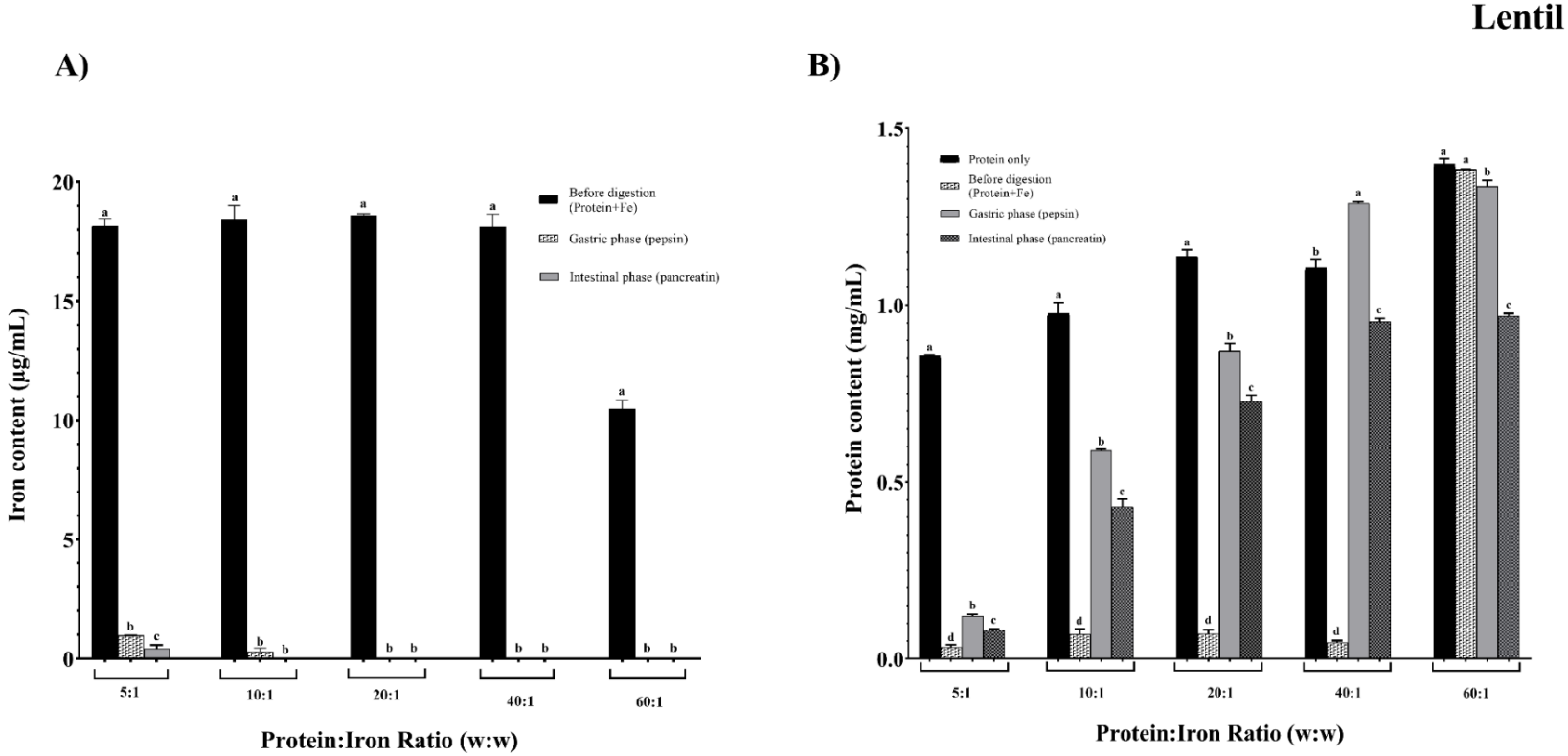

Figure 6.8. (cont.)

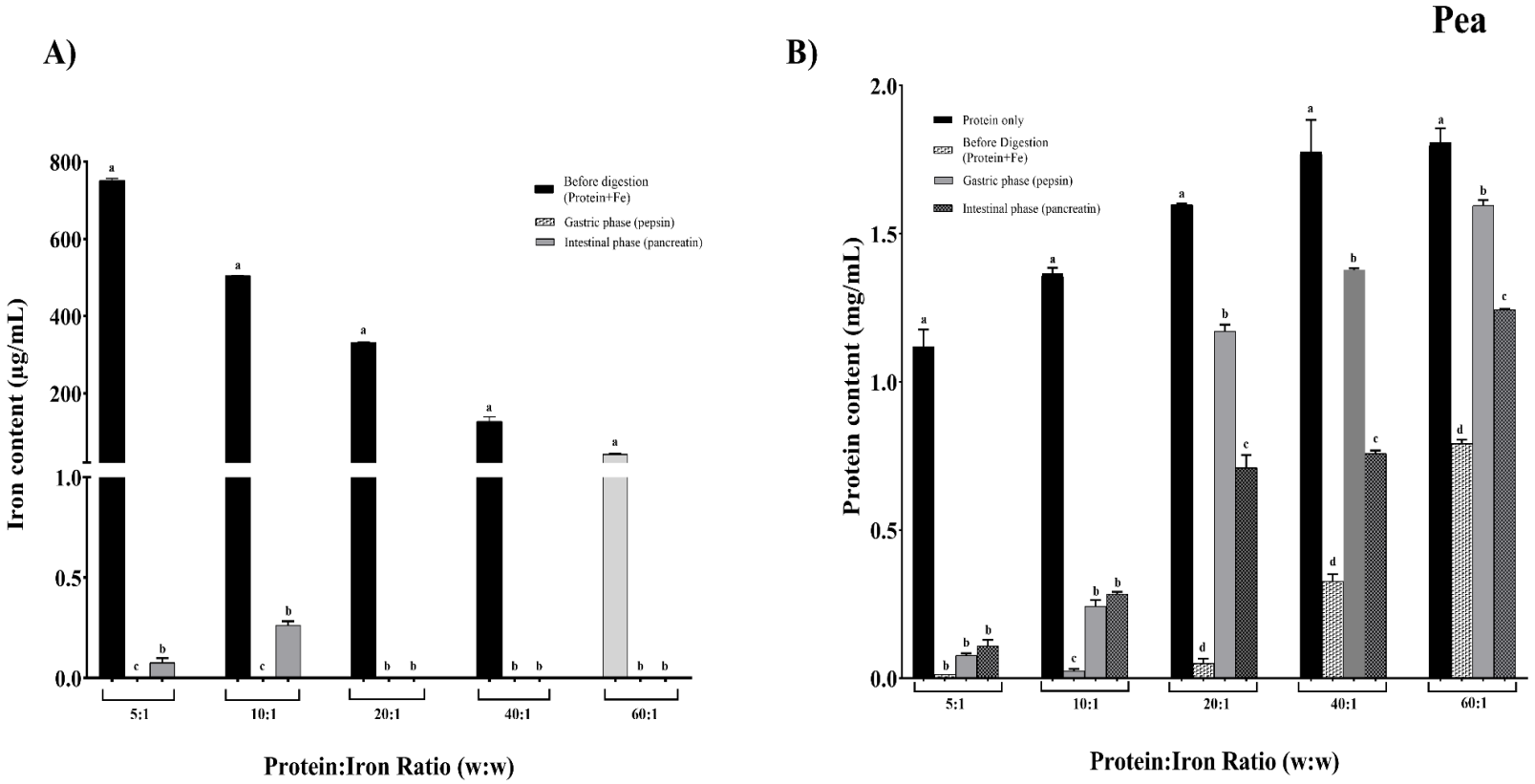

Figure 6.8. (cont.)

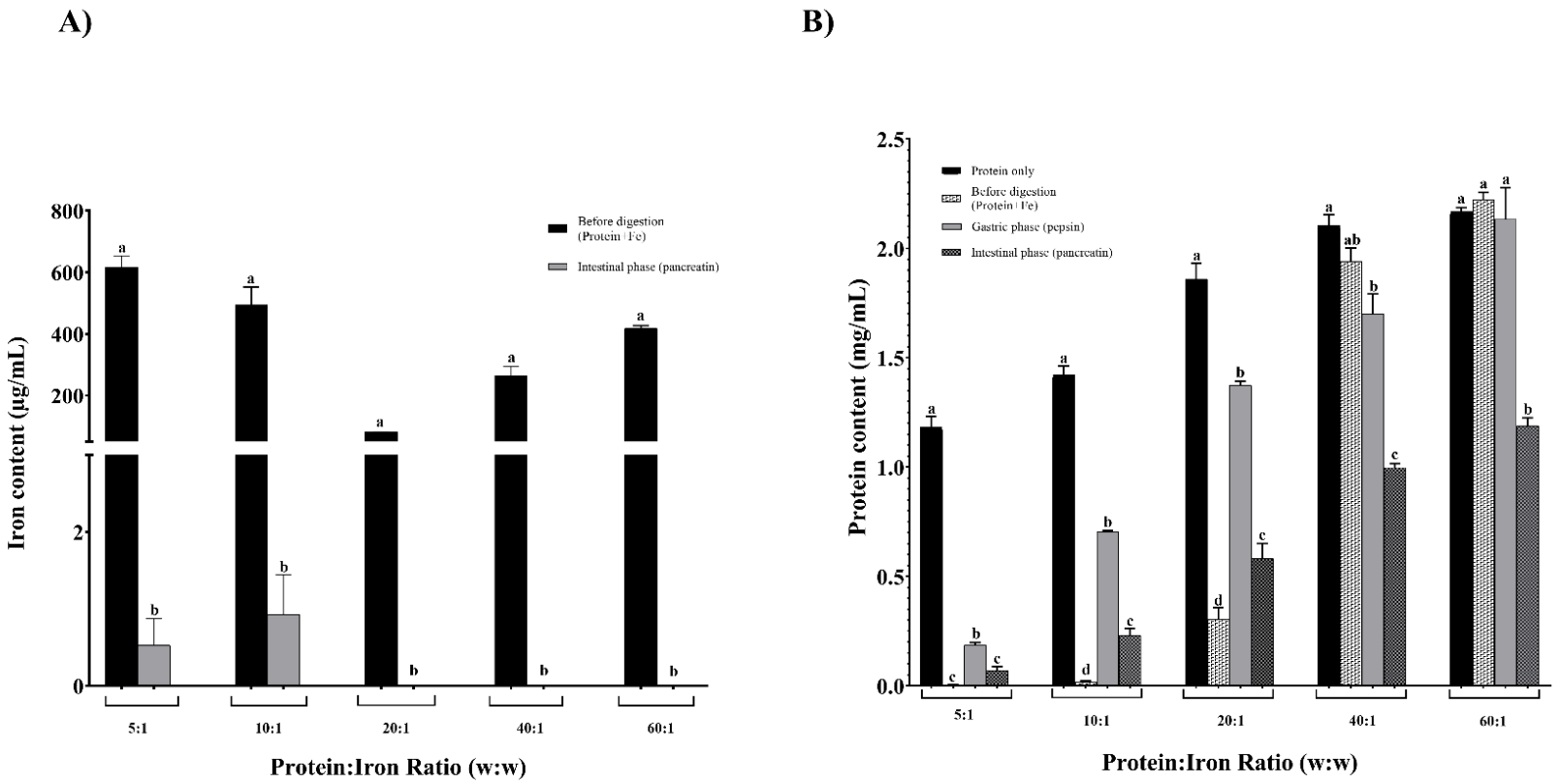

Figure 6.8. (cont.)

functions. Another critical point was that when protein extract was incubated with iron (without digestion), it was observed that there was unbound residual free iron in the solutions at all protein:iron ratios between 0-60 min time points (Figure 6.8B). However, after pepsin and pancreatin digestion of protein-iron complexes obtained at ratios of 10:1 to 60:1, free unbound iron was not detected, indicating that digestion enhanced iron binding capacity of digested protein in solutions, most likely by the production of peptides. For chickpea, although the ratio of 40:1 did not contain free iron mineral in the gastric phase, some amount was detected in the environment after intestinal digestion. So, the ratio of 60:1 was chosen for cell experiments that contained no free iron. When soybean was examined in terms of the free iron amount after digestion, it was observed that the ratios from 20:1 to 60:1 were suitable for cell experiments. The lowest and highest ratios (20:1 and 60:1) were chosen for the cell treatments to see if there would be an effect on the functionality of having too much protein in the environment. For pea, the ratio of 40:1 and 60:1, as low and high concentrations for the same intend soybean, were chosen for cell experiments that contained no free iron.

During the intestinal phase (pH 7.5), the soluble protein content of legumes increased up to a point due to the moderate hydrolysis of proteins to peptides as expected (Figure 6.8B). According to the literature, it was reported that the sequential hydrolysis system results in the production of a wider range of peptide sequences with more cut-off sites that might serve as functional metal-binding sites (Polanco-Lugo *et al*., 2014; Sanchez-Chino *et al*., 2018). Miao *et al.* (2019) also showed that moderate hydrolysis is of great importance for iron chelation of casein hydrolysates in terms of exposure of more active sites for bivalent iron-binding.

The stability of protein-iron complexes formed after digestion at different concentrations was also evaluated under simulated *in vitro* digestion conditions. First, only protein extract was digested and at the end of the simulated *in vitro* digestion experiments iron was added (Figure 6.9). It was observed that for all the ratios, free iron remained in the solution after digestion. With this experiment, if there was any change in their iron binding capacities after digestion wanted to be seen with the same amount of initial iron load. However, free iron still remained in the solution after digestion it was decided not to use for further cell studies.

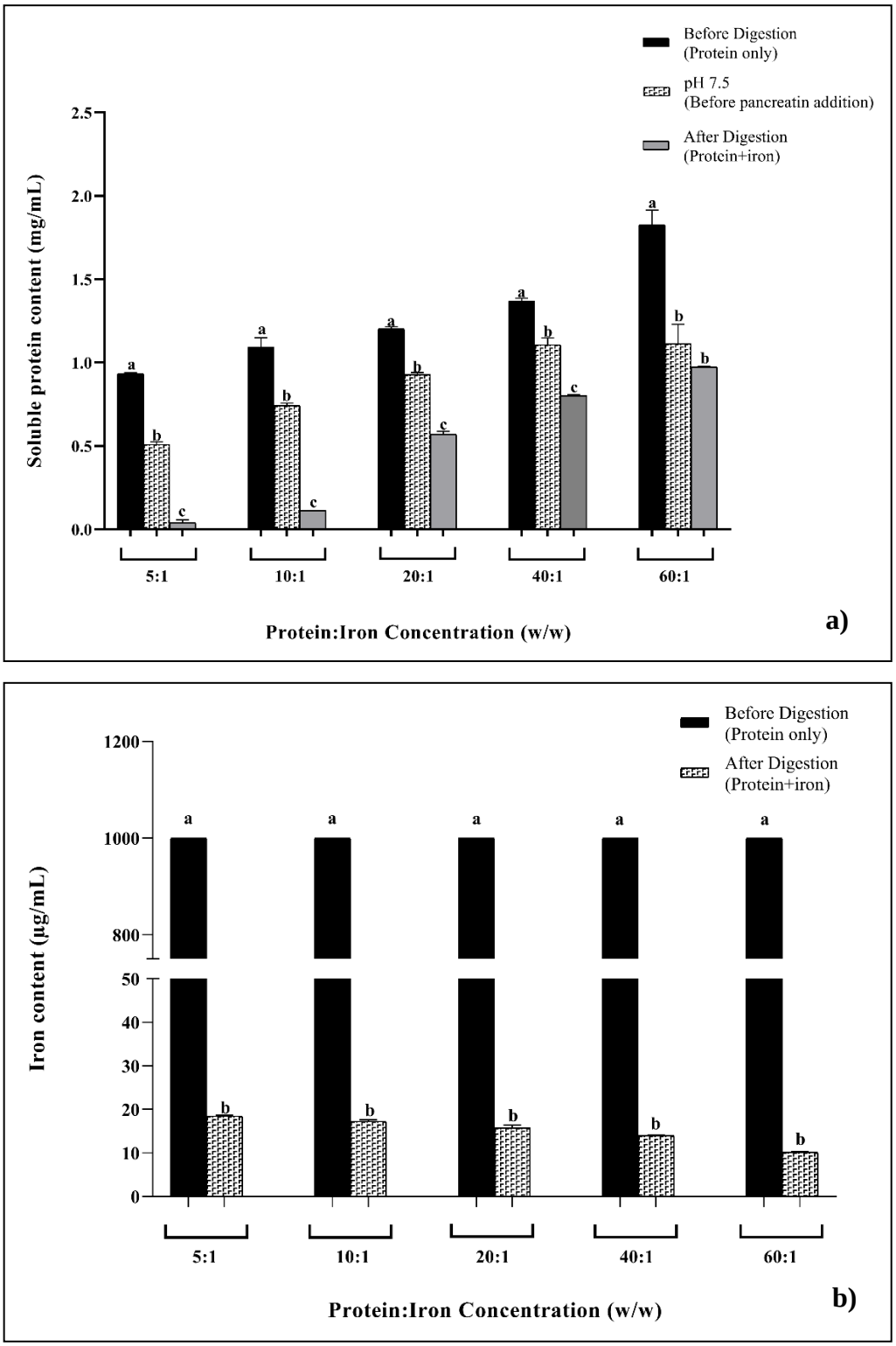

Figure 6.9. a) The soluble protein content of protein-iron complexes b) Bioaccessibility of iron after simulated *in vitro* digestion (Protein-iron complexes formed after digestion) at different concentrations. Bars with different lowercase letters are significantly different ($p < 0.05$). Results are shown as mean ± standard deviation.

6.5.4. Enzymatic Hydrolysis Assay for Protein Extracts of Legumes

Protein extracts (5% protein solution; w/v) were hydrolyzed by the pancreatin enzyme at pH 7.5 and 37ºC for 8h with sampling every hour to determine the suitable hydrolysis time in terms of the iron chelating ability of legumes. Due to the limited solubility and moderate digestion of legume proteins, enzymatic hydrolysis could be effective for changing appropriate physicochemical qualities that can aid intestinal protein absorption without compromising functional quality. (Polanco-Lugo et al., 2014). Iron chelating activities of legumes with regarding time were illustrated in Figure 6.10.

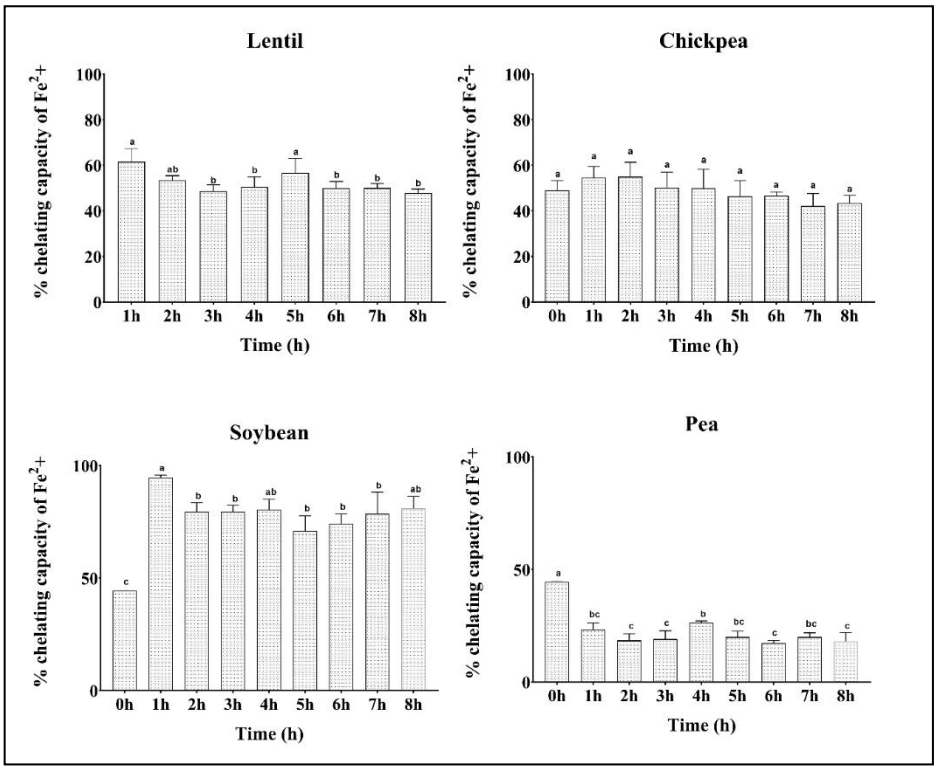

Figure 6.10. Iron-chelating activities of the hydrolyzates of lentil, chickpea, soybean, and pea proteins at different hydrolysis times. Bars with different lowercase letters are significantly different ($p < 0.05$). Results are shown as mean ± standard deviation.

The iron-chelating activities of hydrolysates obtained at different hydrolysis times ranged from 47 to 63%, 42 to 55%, 70 to 94%, and 17 to 26% for lentil, chickpea,

soybean, and pea, respectively at a concentration of 5 mg/mL. These results indicated that the enzyme treatment was effective on the chelating activity. This may be mainly attributed to the molecular weight of peptides, which may take an important role in chelation (Miao *et al.*, 2019). Eckert et al., (2014), showed in their research hydrolysis was an effective method to generate metal binding peptides from barley protein. They showed that highly exposed side chains and higher solubility of hydrolysates ensured more functional binding sites which facilitate interactions and impart stability to the metal-peptide complexes. However, they found no clear relationship between the solubility enhancing effect and hydrolysis time of the peptides. According to the study conducted by Lv et al. (2014), it was shown that the iron bioavailability of protein extract with iron (being hydrolyzed with pancreatin for 4h) is significantly higher than that of intact protein isolate with iron. Moreover, both hydrolysis and fractionation based on molecular weight significantly improved metal ion solubility near neutral pH. In the literature, it was stated that higher solubility of metal ions at acidic pHs (3–5) improves their absorption at the duodenum and proximal jejunum where the mild acidic condition as well as other factors, such as higher level of expression of transport proteins, facilitate the absorption (Collins & Anderson, 2012). Peptides of high chelating capacity can improve a mineral's solubility at neutral pH in the small intestine (Eckert *et al*., 2014).

6.6. Conclusion

(i) Three different simulated gastrointestinal digestion assays were applied to lyophilized legume protein extracts and the methods were compared to find out convenient digestion method.

(ii) Method 1 was chosen for further optimization studies considering the digestion efficiency and its lack of interference with iron ions.

(*iii*) Optimization of simulated gastrointestinal digestion was performed according to the Box-Behnken experimental design. Maximum soluble protein (92.4 mg/g) was obtained at 7% and 4% of pepsin and pancreatin concentration, respectively with 2-hour gastric digestion.

(*iv*) To fix the digestion parameters, numerical optimization was applied. 8% (w/w) for pepsin concentration, 4% (w/w) for pancreatin concentration with 2-h for gastric incubation were fixed as optimal conditions for higher digestion efficiency.

(v) Iron binding profiles of legumes were generated with different ratios during the complexation period. The iron amount was constant at the ratios, whereas protein amount was increased gradually to understand the effect of protein amount on iron chelation. As protein amount increased iron amount decreased, indicating the chelation. However, after 30-min interaction, no major changes were observed in the iron amount considering all the legumes. The overall binding results suggested that most of the iron binding was completed within 30 min. Complexes might suffer instability or iron oxidation if the formation time is kept longer. Therefore 30 min was fixed as formation time.

(vi) After protein-iron complexes were formed, simulated *in vitro* digestion studies were carried out with different protein:iron ratios (w/w) as 5:1, 10:1, 20:1, 40:1, and 60:1, respectively under optimized and validated digestion conditions.

(vii) It was significantly important that digest did not contain free iron after digestion to be able to make sure that functionality if it was, comes from the complex, not from the free form. So, for chickpea, the ratio of 60:1 was selected for further cell experiments. Because this was the ratio that contained no free iron at the end of the digestion. Although ratio of 40:1 did not contain free iron in the gastric phase, some amount was released after intestinal digestion. When examined the protein contents of complexes, protein amount decreased when the iron bound to the protein. As digestion continued, protein amount was gradually increased during digestion. The same trend was followed for other legumes.

(viii) At a ratio of 10:1 was the first ratio that iron was not been detected in the solution after digestion for lentil protein-iron complexes. Hence this ratio was selected for cell experiments because it was thought that minimizing digested protein amount at the minimum level could be important in terms of cellular function. Within this context at a ratio of 60:1 was also selected to compare the cellular functionality.

(ix) For pea-derived protein-iron complexes, 20:1 and 60:1 ratios were selected as the lowest and the highest for cell experiments.

(x) When soybean was examined in terms of the free iron amount at the end of the digestion, it was observed that the ratios of 20:1 and 60:1 were suitable for the cell experiments.

CHAPTER 7

DETERMINATION OF THE EFFECTS OF PROTEIN-IRON COMPLEXES ON IRON METABOLISM AT THE MOLECULAR AND GENETIC LEVEL

Food fortification with iron, which is the addition of iron to processed foods, is the most practical, cost-effective, long-term, and food-based approach in order to overcome iron deficiency anemia (IDA). The World Health Organization (WHO) has also recommended it as a significant method for preventing micronutrient deficiencies (FAO/WHO, 2007). However, it has several iron-related limitations such as limited solubility, oxidative processes such as lipid oxidation, and flavor and color change in iron-fortified food. Low dietary iron bioavailability is the most effective factor that causes IDA among these limitations. (Caetano-Silva et al., 2018). As a result, new alternative ways for iron fortification have been developed that provide food with minimum physical and sensory alteration with high bioavailability.

Food, nutrition, and health are all becoming increasingly important to consumers, especially in recent years. Food is designed to not only relieve hunger and provide important nutrients, but also help avoid nutrition-related illnesses, minimize health risks, and enhance human well-being. (Admassu et al., 2018). Natural compounds, such as bioactive peptides, are becoming more popular as a result of this greater awareness (Lafarga and Hayes, 2017). Peptides derived from food protein extracts or hydrolysates have recently attracted interest as novel metal chelators. Chelated minerals are less prone to chemical reactions with the environment and are more stable (Caetano-Silva et al., 2018), with an equivalent or better effect compared to inorganic salts alone (Eckert et al., 2016). Also, amino acids and other organic acids help to improve iron absorption by buffering the pH of the intestinal contents. (Torres-Fuentes et al., 2012). The use of iron-protein or iron-peptide complexes as an innovative strategy to solve the issues associated with iron fortification might be a feasible alternative.

Legume proteins extracts are commonly utilized as functional components; however, their functionality must be proved in a cell culture system by assessing their physiological activity. Therefore, understanding how the mechanism works is critical. Within this context, the objective of this chapter of the thesis was the formation of protein-iron complexes from protein extracts of legumes and to investigate the effects of these complexes after *in vitro* simulated digestion on iron bioavailability within the Caco-2 cell model. The potential of using these complexes to enhance iron absorption in the small intestine was tried to be evaluated. The experimental steps applied are shown in Figure 7.1 below.

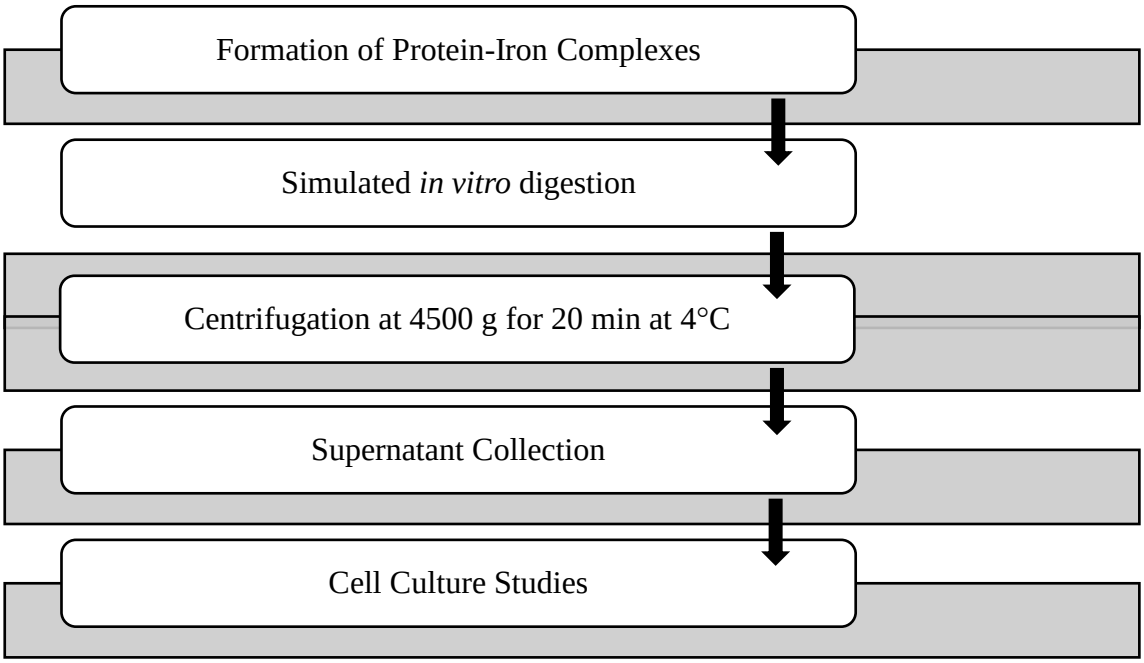

Figure 7.1. Flowchart of design of experiments.

7.1. Induction of Iron Deficiency Anemia and Treatment of Digested Protein-iron Complexes in Caco-2 Cells

For the investigation of the effects of protein-iron complexes on molecular and genetic regulation of iron metabolism on anemic cells, Caco-2 cells were seeded at 1×10^5 cells/well into classical cell culture plates (12-well plates) (Costar, Cambridge, MA). Optical microscopy was used to confirm the monolayer's integrity. Iron deficiency

anemia was induced in the cells at 10-day of post-seeding using a chemical agent (Deferoxamine, DFO) at a concentration of 200 µM. After incubation with DFO agent for 24 h, anemic cells were treated with either iron ($FeSO_4.7H_2O$); 100 µg/mL) or protein-iron complexes for further18-hour-incubation in the incubator with specified concentrations (protein:iron ratio (w/w) which contained no free ferrous iron. The experimental procedure was summarized in Figure 7.2.

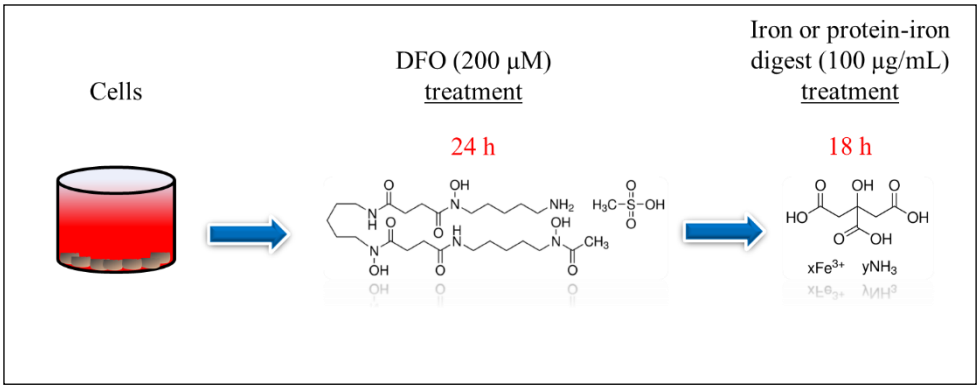

Figure 7.2. Induction of iron deficiency and subsequent treatments with iron or protein-iron complexes in cells growing on 12-well plates and 12-well inserts.

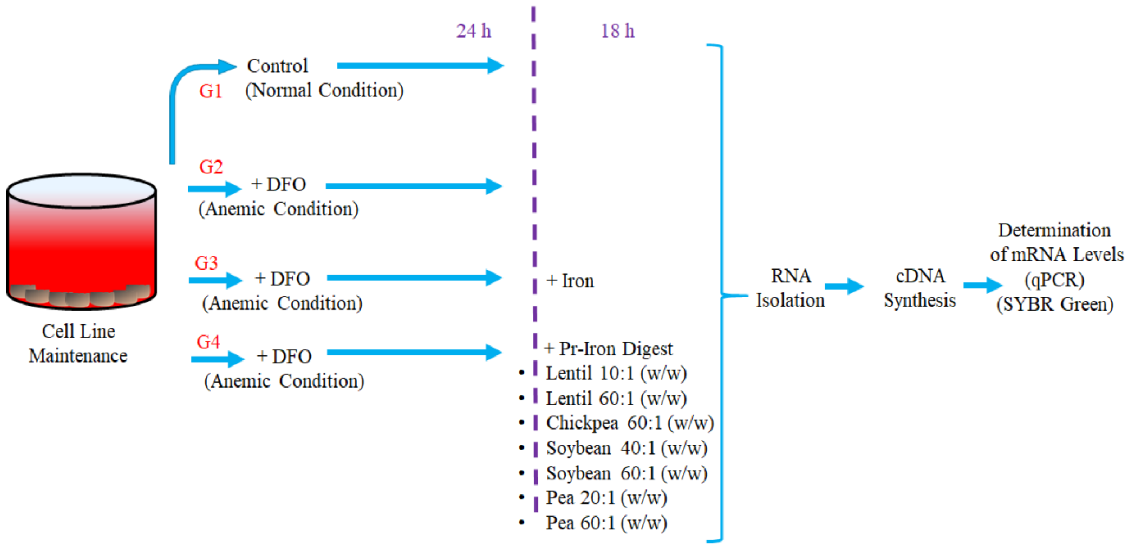

Figure 7.3. Determination of the effect of iron mineral on the molecular and genetic regulation of iron metabolism in anemic cells growing on 12-well plates. G1: Control group, G2: Anemic group, G3: Iron treated group in anemic condition, G4: Digest of protein-iron complexes treated groups in anemic conditions.

7.2. Total RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

The levels of mRNA expression of marker genes involved in iron metabolism and hypoxia were assessed. Total RNA was extracted from cells using the RNAzol reagent (MRC, Cat. No.: RN190), according to the manufacturer's instructions. Using the cDNA synthesis kit, one microgram of each total RNA was transformed to cDNA (Lifetech, Cat. No.: 4368814). Gene-specific oligonucleotide primers and SYBR-Green mix (Lifetech, Cat. No.: 4367659) were used in qRT-PCR using an ABI StepOnePlus instrument (Lifetech, CA, USA) by using. C_T (threshold cycle) levels were normalized according to human cyclophilin A (CypA) mRNA expression as a housekeeping gene. Mean fold changes in gene-specific mRNA levels from all experimental groups were calculated by the $2^{-\Delta\Delta Ct}$ analysis method (Gulec and Collins, 2013).

Table 7.1. Marker genes that were used in the evaluation of the effect of treatments on iron metabolism

Iron Metabolism	Hypoxia
DMT1	*ANKRD37*
TFR	
FPN1	

7.3. Statistical Analysis

Data were statistically evaluated by one-way analysis of variance (ANOVA) using the PRISM software, version 6 (Graph Pad Software, Inc., San Diego, CA, USA). Significant differences between means were determined by Tukey's Multiple Comparison Test procedure at the 5% significance level. The results were considered statistically significant for *p* values less than 0.05.

7.4. Results and Discussions

7.4.1. Cell Studies – Effect of Digested Protein-Iron Complexes on Iron Dependent Gene Regulation

Body iron homeostasis is regulated by intestinal iron absorption because mammals lack active excretion systems (Gulec and Anderson, 2014). The enterocytes cells are important for nutritional absorption, and they make up around 95% of all intestinal cell types (Shirazi-Beechey *et al.,* 2011). The physiology of human enterocyte cells has been modeled with very different cell lines. Caco-2, the collateral adenocarcinoma cell line, has been utilized to study iron metabolism because it responds well to iron deficiency anemia in *in vitro* (Alvarez-Hernandez *et al*., 1998; Linder *et al*., 2003; Hu *et al*., 2010; Gulec *et al*., 2018). DFO treatment induces mRNAs levels of iron regulating genes including divalent metal transporter 1 (*DMT1*, function: to uptake dietary iron into enterocyte cells), transferrin receptor (*TFR*, function: iron transport), and ankyrin repeat domain 37 (*ANKRD37*, function: hypoxia regulating gene). They are highly sensitive genetic indicators for iron metabolism and their mRNA levels were induced DFO dependent iron deficiency anemia (Hu *et al*., 2010). Intestinal hypoxia-inducible factor 2α (HIF-2α) which is a transcriptional factor, is essential for iron absorption during iron deficiency by regulating apical and basolateral iron transporters (Schwartz *et al.*, 2019). Furthermore, the *ANKRD37* gene is regulated by HIF-2α protein in Caco-2 cells under DFO and iron treatment.

In this final part of the thesis related to food science and nutrition, firstly, the effect of iron mineral on the molecular and genetic regulation of iron metabolism in anemic cells growing on 12-well plates was investigated. Iron deficiency anemia was induced using a chemical agent, DFO, which is a very common molecule used for anemic conditions in iron metabolism by causing iron unavailable for cells in medium, and consequently anemic phenotype occurs on cells (Zerounian and Linder, 2002; Hu et al., 2010). When the cells reached 10-day-confluency, they were treated with DFO and then iron, and protein-iron complexes that obtained digestion. Ferrous sulfate heptahydrate ($FeSO_4.7H_20$) form, that conforms to the physiological conditions was used as the iron source in the experiments based on the previous studies in the literature (Hu et al., 2010).

Thereafter, as described in previous sections, RNA isolation, cDNA synthesis, and subsequent qPCR method were applied for the target genes mentioned.

Functional features of protein:iron complexes were tested on DFO induced iron deficiency anemia. The selected ratios were where the free iron was not detected in the solution after digestion to be able to ensure that the functionality really comes from digested protein-iron complexes and in the case of excessive peptide condition to be able to have an opinion if this excessive amount of peptide may create a physical barrier for iron accessibility of cells treated with digested protein-iron complexes. The selected digested protein-iron complexes are as follows: Lentil protein:iron ratio of 10:1 and 60:1, chickpea protein:iron ratio of 60:1, soybean protein:iron ratio of 40:1 and 60:1, and pea protein:iron ratio of 20:1 and 60:1, respectively. It was intended to test ratios that did not have any free unbound iron with low and high protein levels due to cellular effect on the digested protein (or peptide).

After cell treatment with the peptides released from soybean during simulated gastrointestinal digestion at the ratio of 60:1 inhibited the cell proliferation. It was observed that all cells were dead at the of the treatment. Therefore, this treatment group was eliminated by itself. No lethal effect was observed at the ratio of 40:1 for the same protein, soybean. So, it was concluded that this lethal effect occurs in a dose-dependant manner, and soybean ratios to be used in the cell experiments were updated as 20:1 and 40:1. This was in line with the findings of Gonzáles-Montoya *et al*. (2018) study. They had demonstrated the ability of soybean gastrointestinal digests to prevent the proliferation of human colon cancer cell lines.

As in other parts of the thesis lentil protein extract was chosen as a model therefore the effect of protein-iron complexes derived from lentil proteins on iron metabolism was investigated initially (Figure 7.4). It was used two different protein-iron ratios: 10:1 and 60:1. It was observed that *DMT1* and *TFR* mRNA expression levels increased under iron deficiency anemia induced by DFO compared to the control group. Subsequent reduction of dietary nonheme ferric iron, Dmt1 transports the ferrous iron from the absorptive surface of the small intestine across the apical membrane of enterocytes (Gulec et al., 2014). Our results were in accordance with the previous studies in which it has been reported that the intestinal *DMT1* expression had been strongly upregulated by iron deprivation and consequent hypoxia, accounting for the increased uptake of iron under in deficiency (Linder et al., 2003; Gulec et al., 2014). Transferrin is the plasma iron transport protein and TfR-bound ferric iron is distributed to the circulation throughout the body

(Gulec et al., 2014). It had been shown that TfR expression was increased in response to iron deficiency and decreased when intracellular iron levels were high (Zoller et al., 2002). Therefore, it could be said that an increase in *TFR1* expression was indicative of iron deficiency as we observed in our experiments, except for the complex for 60:1 ratio.

It has also been shown that when DFO was given to Caco-2 cells the level of Hif2α protein increased and this increase caused an increase in the Ankrd37 level (Hu et al., 2010). In this study, the iron mineral that had been administered to the cells reduced the levels of *ANKRD37* mRNA to levels in the control group. Hypoxic condition diminished due to iron mineral given to the cells except for the complex for 60:1 ratio. In other words, hypoxia due to iron deficiency anemia increased the expression of *ANKRD37* mRNA compared to control groups.

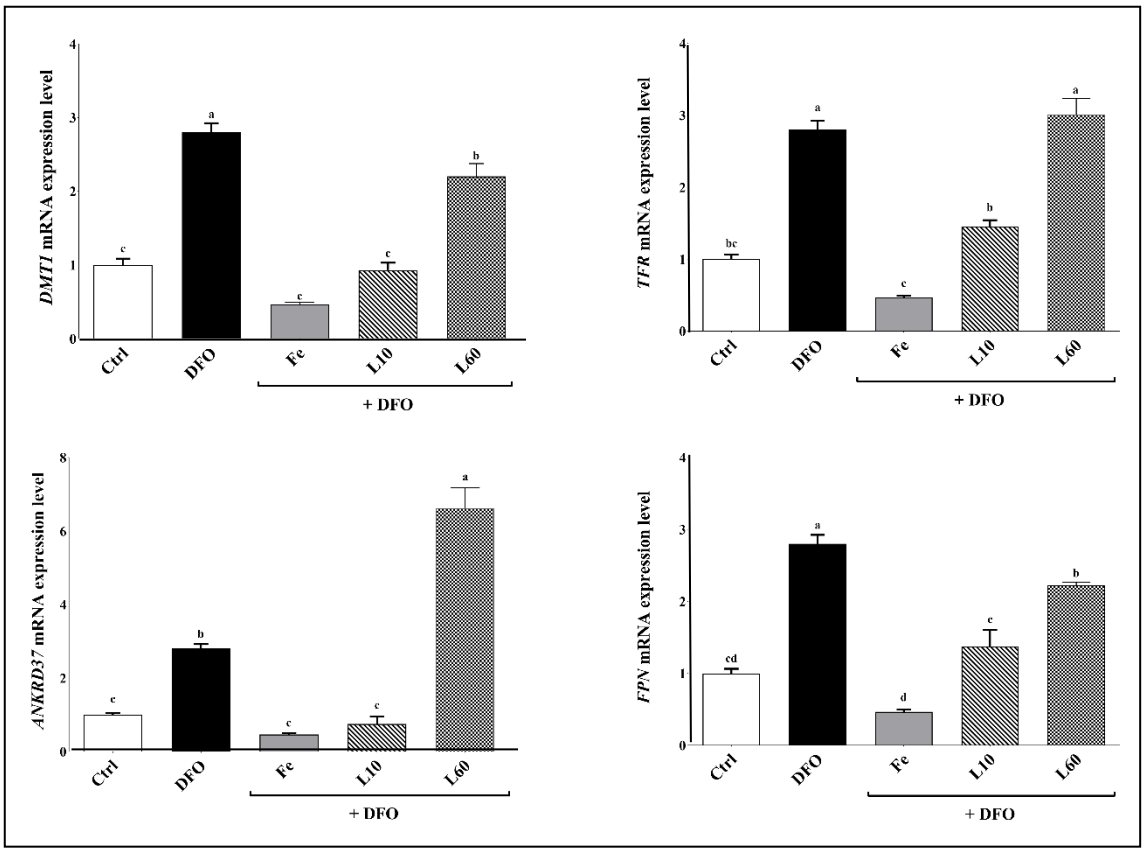

Figure 7.4. The effect of protein-iron complexes on iron metabolism and hypoxia-induced gene expression on anemic cells growing on 12-well inserts. *Tfr*: Transferrin Receptor, *Dmt1:* Divalent metal transporter 1, *Ankrd37:* Ankyrin Repeat Domain 37. Data presented as mean ± SD. Different letters on bars indicated significance ($p < 0.05$). **(cont. on next page)**

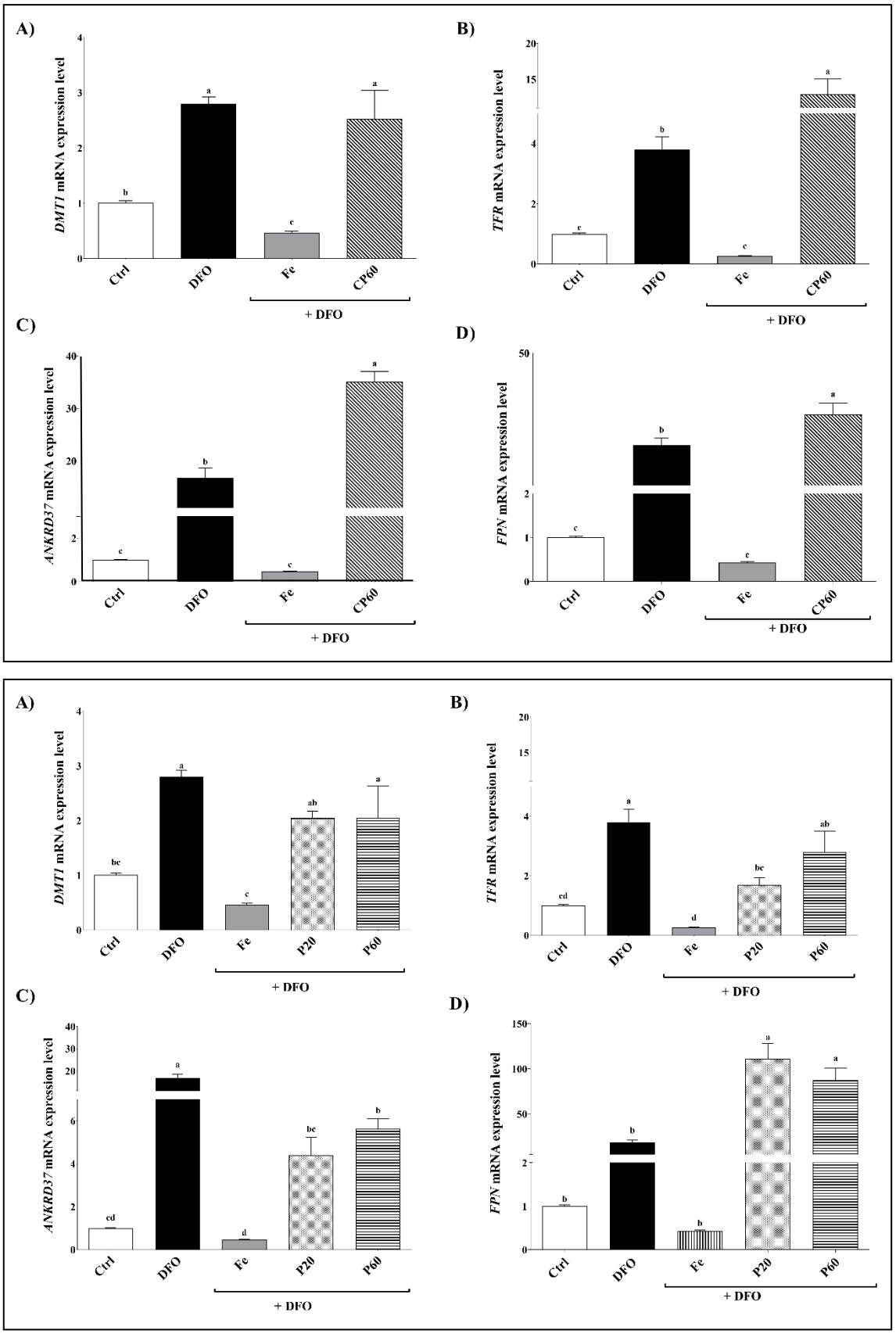

Figure 7.4. (cont.)

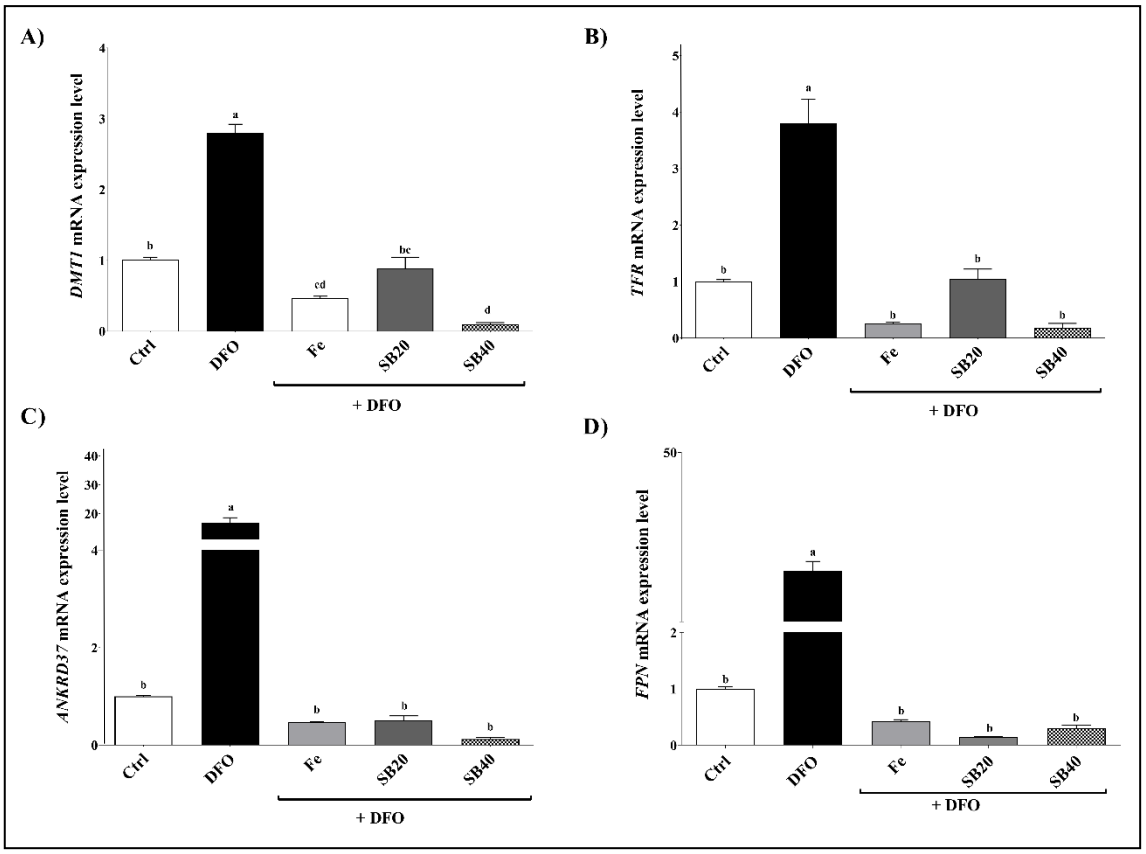

Figure 7.4. (cont.)

The increase of solubility is dose-dependent, which might be related to the occurrence of several binding sites on peptide molecules with varied affinities. Metal ions bind to the sites with the highest affinity at high concentrations, and when they are saturated, they bind to less specific sites with lower affinity (Eckert et al., 2014). This might be the explanation why the concentration of 60:1 did not improve the anemic state.

It was observed that *DMT1*, *TFR*, *FPN*, and *ANKRD37* mRNA expression levels significantly decreased compared to the DFO treated group when anemic Caco-2 cells were treated with peptide–iron complexes for protein–iron ratios of 10:1 for lentil, ratios of 20:1 and 40:1 for soybean peptide-iron complexes and beyond, indicating that digested protein–iron complexes reduced the anemic condition in these cells. However, the same significant reduction was not observed for the lentil ratio of 60:1, all ratios of pea and chickpea peptide-iron complexes (Figure 7.5).

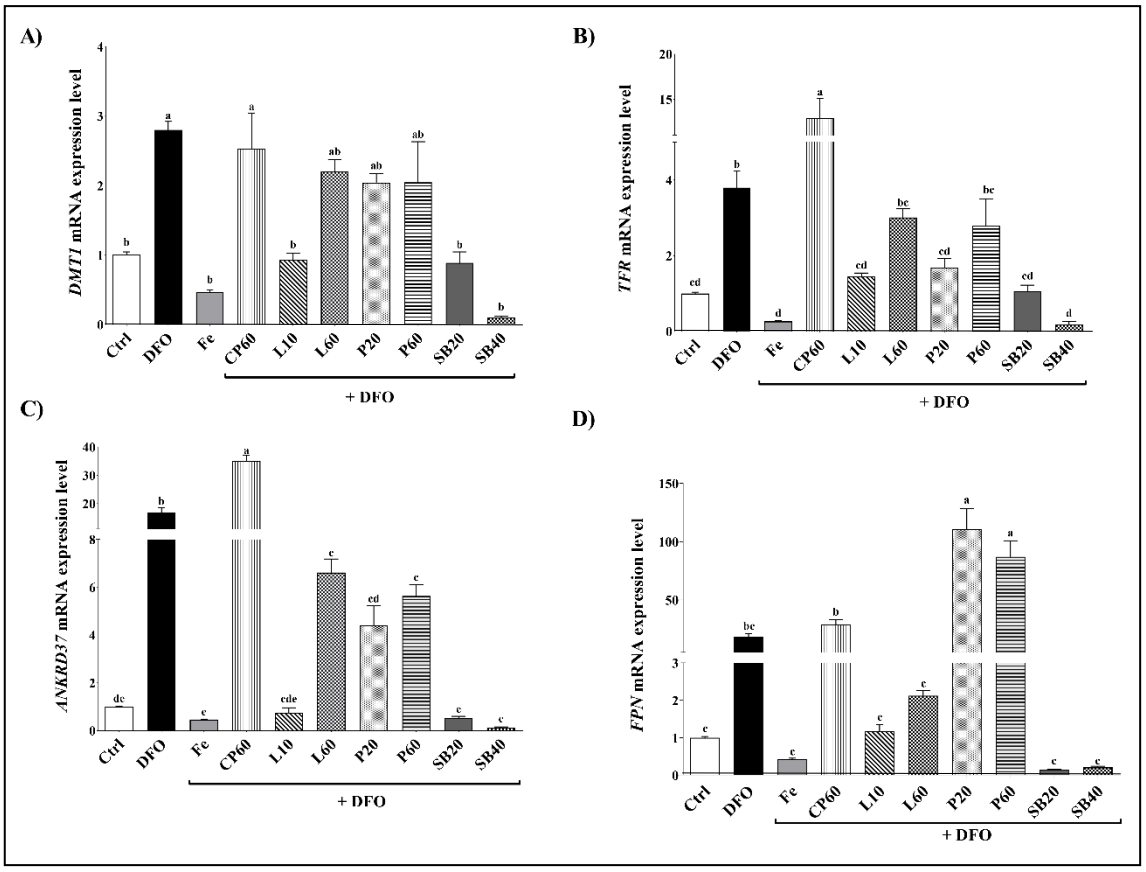

Figure 7.5. The effects of digested peptide–iron complexes derived from legumes on iron metabolism and hypoxia-induced gene expression on anemic cells growing on 12-well inserts. (A) *DMT1*: Divalent metal transporter 1, (B) *TFR*: Transferrin receptor, (C) *ANKRD37*: Ankyrin repeat domain 37 (D) *FPN*: Ferroportin. Data were presented as mean ± standard deviation. Bars with different lowercase letters are significantly different ($p < 0.05$). (Ctrl: Control group, DFO: Deferoxamine-treated group, Fe: Iron-treated anemic cells, CP60: Chickpea pr.:iron complex 60:1 (w/w), L10: Lentil pr:iron complex 10:1 (w/w), L60: Lentil pr:iron complex 60:1 (w/w), P20: Pea pr.:iron complex 20:1 (w/w), P60: Pea pr.:iron complex 60:1 (w/w), SB20: Soybean pr.:iron complex 20:1 (w/w), SB40: Soybean pr.:iron complex 40:1 (w/w)).

There might be explained in two ways that the complexes with high protein concentrations did not have any effect on gene regulation. First, the hydrolyzation of high protein-iron complexes might cause excessive production of free peptides, and these free peptides might interfere with the absorption of the iron–peptide complexes into cells, or secondly, high amounts of peptides might be reduced the molecular effects of the iron

onto the cells. Furthermore, peptides of pea and chickpea that showed no molecular effect on iron deficiency anemia could be concentrated through ultrafiltration and then the cells could be treated with these complexes. However, these hypotheses must be tested by further experiments.

Peptide absorption mechanisms through intestinal epithelial cells are complicated. So far, two basic mechanisms have been proposed. Although proteins can be hydrolyzed into amino acids in the epithelial cells, one of the proposed mechanisms is the transportation of di- and tripeptides by a specific transporter named PepT1. The second mechanism is passive transport via the paracellular pathway, which has been proposed as the principal mechanism for transporting intact peptides across the small intestine's monolayer of epithelial cells (Lafarga and Hayes, 2016). So, PepT1 should be evaluated as a marker gene for further studies. Also, the amount of protein in the cells should be detected with appropriate methods. According to the findings of Zhu et al. (2009), the ligands do not form a physical barrier or chelate iron with a high enough affinity to prevent iron absorption by the cells (Zhu et al.,2009). Concerning iron, Lin et al., found that chelating hydrolyzed proteins from hairtail fish species given to anemic rats had an effect and increased hemoglobin and ferritin concentrations; however, there was no difference with ferrous sulfate alone. The available data clearly show the enhancing effects of hydrolyzed proteins and peptides on calcium and iron in cellular and animal models. The actual mechanism is rarely investigated and therefore remains largely unknown.

Our results suggested that the physiological effect of peptide–iron complexes on iron regulating gene mRNA expression might depend on peptide concentration *in vitro*. According to Eckert et al. (2014), various barley-derived peptide compounds could have diverse binding capacities to a range of metal ions. Peptide and mineral interactions have also been studied in other protein sources, including chickpeas, fruit drinks, and barely, and with the conclusion that peptides from these sources lower iron and calcium mineral toxicity while simultaneously increasing mineral bioavailability. Furthermore, the synthesized counterpart of the heptapeptide increased iron bioavailability in Caco-2 cells, demonstrating that iron binds to the peptide in an amino acid-specific way. Because free iron causes toxicity in cells, the stability of the peptide–iron complexes is critical throughout digestion and absorption. Specific peptides are thought to boost iron absorption by enhancing their solubility. Proposed processes include paracellular and/or peptide transporter-mediated insertion of insert peptides and iron into enterocyte cells.

Lin et al. (2015) discovered that giving anemic rats chelating hydrolyzed proteins from hairtail fish species enhanced hemoglobin and ferritin levels. It has also been proven that adding iron–whey peptide complexes into food products improves sensory qualities while lowering the risk of free iron toxicity. These results indicate that peptides increase iron bioavailability and that peptide-mediated iron chelation lowers the toxicity of free iron.

7.5. Conclusion

The formation of protein-iron complexation was carried out and the stability of protein-iron complexes was evaluated under simulated *in vitro* digestion conditions. Then, the effect of protein-iron complexes on iron metabolism was investigated. It was used predetermined protein-iron ratios: Chickpea pr.:iron complex 60:1 (w/w) as CP60, lentil pr:iron complex 10:1 (w/w) as L10, lentil pr:iron complex 60:1 (w/w) as L60, pea pr.:iron complex 20:1 (w/w) as P20, pea pr.:iron complex 60:1 (w/w) as P60, soybean pr.:iron complex 20:1 (w/w) as SB20, soybean pr.:iron complex 40:1 (w/w) as SB40, and soybean pr.:iron complex 60:1 (w/w) as SB60. However, cell treatment with the peptides released from soybean during simulated gastrointestinal digestion at the ratio of 60:1 inhibited cell proliferation. It was observed that all cells were dead at the of the treatment. Therefore, this treatment group was eliminated by itself.

It was observed that *DMT1* and *TFR* mRNA expression levels increased under iron deficiency anemia induced by DFO compared to the control group. It had been shown that *TFR* expression was increased in response to iron deficiency and decreased when intracellular iron levels were high. Therefore, it could be said that an increase in *TFR* expression was indicative of iron deficiency as we observed in our experiments. Iron bioavailability of protein-iron complexes of lentil (10:1 ratio) and soybean (20:1 and 40:1 ratios) in the Caco-2 cells were as high as the iron salts. Digested protein (peptide)–iron complexes of lentil and soybean significantly reduced to *DMT1*, *TFR*, *FPN*, and *ANKRD37* mRNA expression levels compared to the DFO treated group, indicating that peptide–iron complexes influence gene regulation in enterocyte cells. However, it is unknown how the peptide–iron complexes were taken into the cells and how they affect the cellular mechanisms as complexes or dissociated forms. The actual mechanism, which is rarely investigated, is worth exploring in the future. To the best of our knowledge, this is the first study that shows the functionality of legume peptide–iron complexes in a

nutrition-associated iron deficiency anemia model in the cell culture system. Our cell culture results should be tested in animals or human models to show systemic effectiveness of peptide–iron complex against iron deficiency anemia. Even as it is, protein-iron complexes are promising agents for use in food fortification.

CHAPTER 8

CONCLUSION

In this Ph. D. thesis, the compensatory effect of copper mineral and protein-iron complexes on iron deficiency anemia in human enterocyte cell culture model were investigated. A nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from the diet. If iron intake is limited or inadequate due to poor dietary intake, or in the presence of some chronic diseases iron deficiency anemia may occur. Thus, the iron mineral that comes from the diet is important and the intestine plays a vital role to maintain iron homeostasis in the human body. However, whether dietary iron or blood iron level is determinant in the intestinal iron metabolism is unknown. Within this context, the Caco-2 cells were grown in classical cell culture plates (12-well plates) and special bicameral cell culture insert systems for 21 days and investigated the effects of iron mineral on iron metabolism and hypoxia in case of iron deficiency anemia. When comparing the levels of DFO and iron-treated cells grown on plates to cells grown on inserts, it was observed that there was a significant difference in the regulation of *DMT1*, *FTN,* and *HEPH* genes. So, it can be concluded that the level of iron in the blood might be more important than the dietary intake. However, the most important point that should not be missed was that blood iron level was regulated with dietary iron. There are three ways to maintain iron homeostasis in the human body: iron can be injected into one of the blood vessels, iron pills can be used as supplements or, by the development and consumption of functional foods that have high iron bioavailability. Frequent injection of iron is inconvenient and considering the toxicity of iron, it can cause administration problems. Also, some problems that come from taking pills are common such as their side effects (constipation, diarrhea, sickness, vomiting, etc.) and swallowing pills can be a problem, either. Therefore, it is important to develop edible natural iron supplements that can be used as an additive to food products to make them “functional” via enhancing their iron bioavailability in order to reduce iron deficiency risk in humans. With this

study, it was shown that the mechanism of why we need to develop a functional food (Chapter 4).

The total protein contents of lentil, pea, chickpea, and soybean were 0.86, 0.75, 0.60, and 0.62 g/g, respectively. For all the protein extracts, a characteristic U-shaped solubility curve was obtained. In general, the highest solubility was observed at pH 2.0 and the pH ranging between 7-8, indicating the increasing solubility on either side of the isoelectric point. Lentil extract had the highest solubility at physiological pH (pH 7.0), whereas the water-soluble contents of chickpea, pea, and soybean did not show statistically significant differences ($P > 0.05$). The results of Fe^{2+} chelating capacity indicated that the ferrozine assay for the lentil protein extract at physiological pH worked well, in good agreement with the results obtained with Fe^{2+} chelating capacity of the standard at the same pH. Ferrozine used in this assay was unstable at low acidic conditions, especially in pH values less than 4. It was observed that phosphate was a good iron chelator, so phosphate buffer was not suitable for the experiments related to the iron binding capacity determination. For different pH environments, ferrous sulfate as an iron source in place of ferrous chloride seemed to be suitable with this way of use considering the aim of the thesis. Ferrous ion chelating (FIC) abilities of all crude protein extracts were assessed with the use of ferrous chloride and ferrous sulfate solution as an iron source. For both iron solutions used, lentils showed the highest FIC ability followed by soybean pea, and chickpea, respectively (at the concentration of 1500 µg protein per reaction mixture) (Chapter 5).

Three different simulated gastrointestinal digestion assays were applied to lyophilized legume protein extracts and the methods were compared to find out convenient digestion method. Method 1 was chosen for further optimization studies considering the digestion efficiency and its lack of interference with iron ions. Optimization of simulated gastrointestinal digestion was performed according to the Box-Behnken experimental design. Maximum soluble protein (92.4 mg/g) was obtained at 7% and 4% of pepsin and pancreatin concentration, respectively with 2-hour gastric digestion. In order to fix the digestion parameters, numerical optimization was applied. 8% (w/w) for pepsin concentration, 4% (w/w) for pancreatin concentration with 2-h for gastric incubation were fixed as optimal conditions for higher digestion efficiency. Iron binding profiles of legumes were generated with different ratios during the complexation period. The iron amount was constant at the ratios, whereas protein amount was increased gradually to understand the effect of protein amount on iron chelation. As protein amount

increased iron amount decreased, indicating the chelation. However, after 30-min interaction, no major changes were observed in the iron amount considering all the legumes. The overall binding results suggested that most of the iron binding was completed within 30 min. Complexes might suffer instability or iron oxidation if the formation time is kept longer. Therefore 30 min was fixed as formation time. After protein-iron complexes were formed, simulated *in vitro* digestion studies were carried out with different protein:iron ratios (w/w) as 5:1, 10:1, 20:1, 40:1, and 60:1, respectively under optimized and validated digestion conditions. It was significantly important that digest did not contain free iron after digestion to be able to make sure that functionality if it was, comes from the complex, not from the free form. So, for chickpea, the ratio of 60:1 was selected for further cell experiments. Because this was the ratio that contained no free iron at the end of the digestion. Although the ratio of 40:1 did not contain free iron in the gastric phase, some amount was released after intestinal digestion. When examined the protein contents of complexes, protein amount decreased when the iron bound to the protein. As digestion continued, protein amount was gradually increased during digestion. The same trend was followed for other legumes. At a ratio of 10:1 was the first ratio that iron was not been detected in the solution after digestion for lentil protein-iron complexes. Hence this ratio was selected for cell experiments because it was thought that minimizing digested protein amount at the minimum level could be important in terms of cellular function. Within this context at a ratio of 60:1 was also selected to compare the cellular functionality. For pea-derived protein-iron complexes, 20:1 and 60:1 ratios were selected as the lowest and the highest for cell experiments. When soybean was examined in terms of the free iron amount at the end of the digestion, it was observed that the ratios of 20:1 and 60:1 were suitable for the cell experiments (Chapter 6).

The formation of protein-iron complexation was carried out and the stability of protein-iron complexes was evaluated under simulated *in vitro* digestion conditions. Then, the effect of protein-iron complexes on iron metabolism was investigated. It was used predetermined protein-iron ratios: Chickpea pr.:iron complex 60:1 (w/w) as CP60, lentil pr:iron complex 10:1 (w/w) as L10, lentil pr:iron complex 60:1 (w/w) as L60, pea pr.:iron complex 20:1 (w/w) as P20, pea pr.:iron complex 60:1 (w/w) as P60, soybean pr.:iron complex 20:1 (w/w) as SB20, soybean pr.:iron complex 40:1 (w/w) as SB40, and soybean pr.:iron complex 60:1 (w/w) as SB60. However, cell treatment with the peptides released from soybean during simulated gastrointestinal digestion at the ratio of 60:1 inhibited cell proliferation. It was observed that all cells were dead at the of the treatment.

Therefore, this treatment group was eliminated by itself. It was observed that *DMT1* and *TFR* mRNA expression levels increased under iron deficiency anemia induced by DFO compared to the control group. It had been shown that *TFR* expression was increased in response to iron deficiency and decreased when intracellular iron levels were high. Therefore, it could be said that an increase in *TFR* expression was indicative of iron deficiency as we observed in our experiments. Iron bioavailability of protein-iron complexes of lentil (10:1 ratio) and soybean (20:1 and 40:1 ratios) in the Caco-2 cells were as high as the iron salts. Digested protein (peptide)–iron complexes of lentil and soybean significantly reduced to *DMT1*, *TFR*, *FPN*, and *ANKRD37* mRNA expression levels compared to the DFO treated group, indicating that peptide–iron complexes influence gene regulation in enterocyte cells. However, it is unknown how the peptide–iron complexes were taken into the cells and how they affect the cellular mechanisms as complexes or dissociated forms. The actual mechanism, which is rarely investigated, is worth exploring in the future. To the best of our knowledge, this is the first study that shows the functionality of legume peptide–iron complexes in a nutrition-associated iron deficiency anemia model in the cell culture system. Our cell culture results should be tested in animals or human models to show systemic effectiveness of peptide–iron complex against iron deficiency anemia. Even as it is, protein-iron complexes are promising agents for use in food fortification (Chapter 7).

The nutrient-dependent regulation of enterocyte cells is central to the intestinal nutrient-sensing mechanism. The basolateral and apical sides of the enterocyte cells are the primary targets to understand nutrient sensing in terms of nutrient overload or deficiency. Furthermore, the polarization of Caco-2 cells also might influence gene regulation. The results obtained from studies of the first experimental chapter of this thesis suggested that intracellular gene regulation was mainly affected by copper treatment in the basolateral side of enterocyte cells during IDA, indicating that blood copper level might have the ability to control the enterocyte iron metabolism at molecular and genetic levels during iron deficiency anemia. The blood copper level might be an important regulator for intestinal iron metabolism during iron deficiency anemia. The main application of this finding might be that copper mineral could be considered to add with iron mineral for functional food development processes or iron supplements to reduce the risk of iron deficiency (Chapter 3).

This Ph.D. thesis aimed to develop "edible protein-iron complexes" derived from legume proteins, which could be used as a nutritional supplement in food products to

reduce the risk of iron deficiency in humans without causing a change in their current diet in order to reduce iron deficiency anemia, which is the first in the world due to nutrient deficiency and seen in all age groups. The results obtained from this thesis study revealed that the chelating capacity of lentil and soybean protein-iron complexes increased after digestion and these complexes fixed the anemia status in anemic enterocyte cells. And to the best of our knowledge, this is the first study to demonstrate the functionality of legume protein-iron complexes in a nutrition-associated anemia model in a cell culture system. For these reasons, an edible natural supplement to be formed using the iron-binding activity of legume proteins is very interesting in terms of medical nutrition. The copper mineral could be considered to add with iron mineral to proteins for functional food development processes or iron supplements to reduce the risk of iron deficiency.

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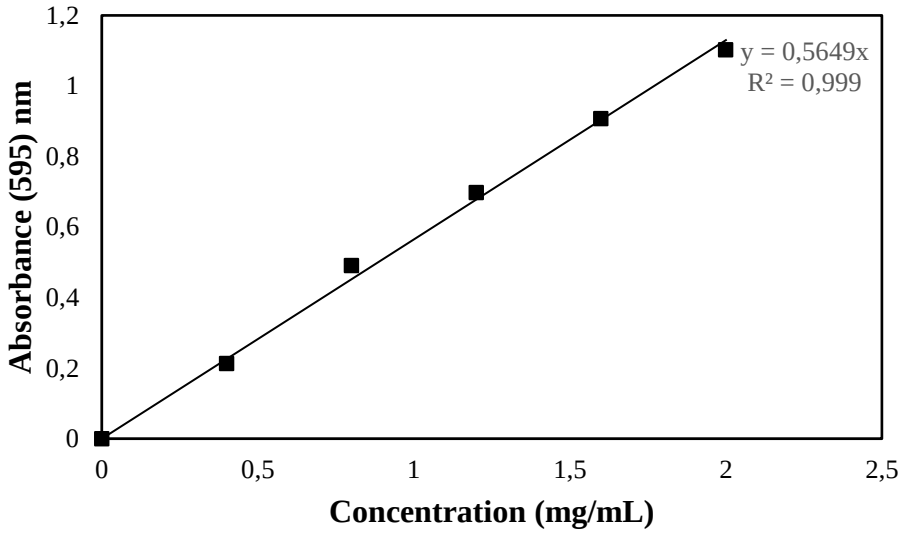
APPENDIX A

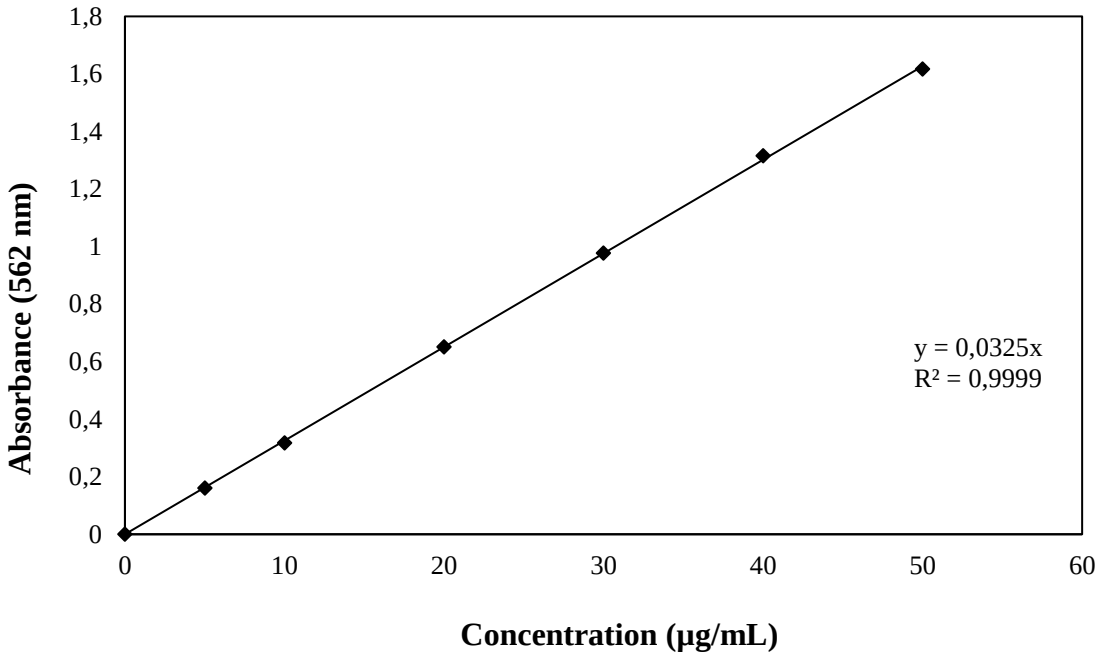
BSA STANDARD CURVE



Appendix A. Bovine serum albumin standard curve for Bradford method.

APPENDIX B

Fe^{2+} STANDARD CURVE



Appendix B. Fe^{2+} ($FeSO_4.7H_2O$) standard curve for ferrozine method.

VITA

Ezgi Hoşer was graduated with a bachelors's degree in the Department of Biology (Basic and Industrial Microbiology) at Ege University, İzmir in 2010. She started the Master programme of the Department of Biotechnology and Bioengineering at İzmir Institute of Technology in September of 2010 and received the Master of Science degree as biotechnologist in June 2012. She studied bioethanol production from apple pomace by using cocultures in her master period. Besides, she worked as a research assistant at Department of Food Engineering in İzmir Institute of Technology between 2010 and 2019. The Food Engineering Department at İzmir Institute of Technology accepted her to study the Philosophy of Doctorate Programme in 2012. At the beginning of her Ph.D., she has gained experience in the field of biotechnology, industrial enzyme production by fermentation technologies as a visiting scholar supported by the European Union Marie Curie FP-7 PEOPLE 2010 IRSES in Argentina at the Center for Research and Development of Industrial Fermentations (CINDEFI CONICET - UNLP). Currently, she has been working on mineral-mineral interaction mechanism and edible functional food production from legume derived proteins using these interactions. During her Ph.D. period, she participated lots of international congresses, conferences, workshops and trainings, as well as presented many oral presentations and posters about her study area. Besides she has two published and one under-review SCI articles throughout her Ph.D. period.