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The development of lentil derived protein–iron complexes and their effects on iron deficiency anemia *in vitro*†

Ezgi Evcan and Sukru Gulec*

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Iron deficiency anemia (IDA) is the most common nutrient-dependent health problem in the world and could be reversed by commercially available iron supplementation. The form of iron supplement is important due to its toxicity on the gastrointestinal system (GI), so the development of new dietary strategies might be important for the prevention of IDA. It has been shown that plant-based proteins bind to iron and might decrease the free form of iron before absorption and increase iron bioavailability. Thus, we aimed to form lentil derived protein–iron complexes and to test the functional properties of hydrolysed protein–iron complexes in anemic Caco-2 cell line. Our main findings were that (i) lentil derived proteins had the capacity to chelate iron minerals and (ii) hydrolysed protein–iron complexes significantly reduced the mRNA levels of iron regulated divalent metal transporter-1 (*DMT1*), transferrin receptor (*TFR*), and ankyrin repeat domain 37 (*ANKRD37*) marker genes that were induced by iron deficiency anemia. The current findings suggest that hydrolysed protein–iron complexes might have functional properties in iron deficiency anemia *in vitro*. Further *in vivo* studies are necessary to show lentil derived proteins and iron might be used as supplements or food additives to reduce the risk of iron deficiency anemia.

1. Introduction

Lentils (*Lens culinaris* L.) are a member of the Leguminosae family and are commonly used in traditional diets. Lentils are produced in many parts of the world. According to the Food and Agriculture Organization of the United Nations Statistical Databases (FAOSTAT), the world lentil production was around 7.5 million tons in 2017, and the top three producers were Canada (1.25 million), India (950 billion), and Turkey (468 billion) between 1994–2017. Lentils are a good source of protein and other micronutrients.[1] Furthermore, the average protein content of lentils is around 21%–31%, with globulins accounting for 70% of the total protein.[2,3]

The relationship between food and diet is very important for maintaining optimal health in humans. Food is not only considered as the source of essential nutrients or a way to prevent hunger anymore, but also expected to provide wellness by preventing nutrient-related diseases.[4] This awareness has given rise to increased interest in natural ingredients, including bioactive peptides.[5] So, there is tremendous attention in peptides of food protein due to their effects on functional health properties, including cell proliferation, inflammation, and metabolic diseases.[6] Some peptides are involved in nutrient–nutrient interactions by chelating minerals,[7] and this might influence 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 its deficiency affects oxygen transport to tissues, cell growth, and energy metabolism.[8]

Iron deficiency anemia (IDA) is the most common nutritional deficiency in the world and affects all age groups in a variety of populations.[9] Commercially available iron supplements are used to reduce 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 GI cells during digestion and absorption. This will ultimately reduce free iron toxicity and increase iron solubility and bioavailability. Iron homeostasis is controlled by intestinal dietary iron absorption because mammals do not have active iron excretory mechanisms.[10] Thus, the enhancement of intestinal iron absorption during IDA is critical to increase the iron level in blood and peripheral tissues.

Molecular Nutrition and Human Physiology Laboratory, Food Engineering Department, İzmir Institute of Technology, İzmir Institute of Technology Food Engineering Department, Room: 215, Urla, İzmir 35430, Turkey.
E-mail: sukrugulec@iyte.edu.tr; Fax: +90232750 6196; Tel: +90232 750 6290

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In this study, we investigated the iron binding ability of lentil derived proteins or hydrolysed proteins along with their functional effects on iron deficiency anemia. The human epithelial colorectal adenocarcinoma cell line (Caco-2) was grown for 10 days and treated with deferoxamine (DFO) to induce iron deficiency anemia. Hydrolysed protein–iron complexes were given anemic Caco-2 cells following the mRNA levels of 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) genes were analyzed to test the role of hydrolysed protein–iron complexes *in vitro*.

2. Results and discussion

2.1. pH Dependent solubility profiles of lentil protein extract and iron salt

The functionality of protein and iron depends on solubility, and pH is one of the important contributors of solubility.[11] Iron salt shows a pH-dependent solubility profile, so it is important to understand its behaviour in different food matrices and under different pH levels during gastrointestinal digestion. Iron must be soluble and stable in its ferrous form (Fe^{2+}) at a higher bioavailability rate in the enterocyte cells of the intestine.[12,13] Thus, the solubility profiles of both lentil protein extract and iron salt were determined under different pH conditions (Fig. 1). For the protein extract, a characteristic U-shaped solubility curve was obtained with peak points between pH 2.0 and 3.0 and between 6.0 and 8.0. As expected, the minimum protein solubility was observed between pH 4.0 and 5.0, which falls within the range of isoelectric point for lentil proteins.[14] In contrast, iron showed maximum solubility at pH 2.0 and 3.0, followed by a sharp reduction in its solubility between pH 3.0 and 6.0. Thus, it is clear that the acidic pH between 2.0 and 3.0 is highly critical, since both protein and iron exist in soluble forms in this range. In contrast, at neutral and close to neutral pH, protein is highly soluble, while iron exists in an insoluble form. Furthermore, amino acids of the protein tend to deprotonate their ionizable electron-donating groups at pH 7.0,[15] and this might enhance the iron binding capacity of amino acids. This can increase iron solubility at neutral pH.

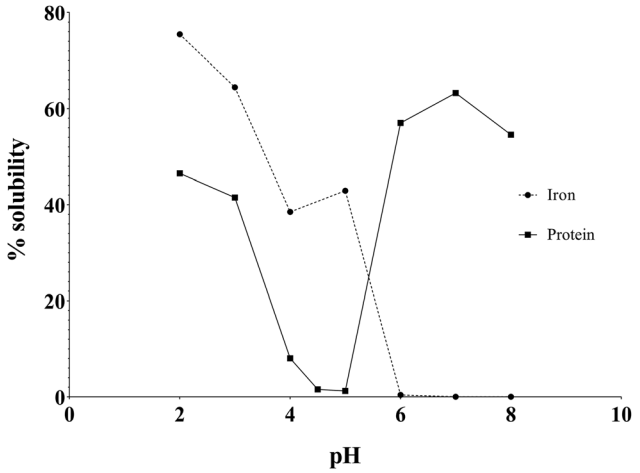

Fig. 1 Solubility profile of lentil protein extract and iron under different pH conditions.

2.1.1. Solubility profiles of lentil protein–iron complexes. The different solubility features of proteins (high) and iron (low) at neutral pH can be used to increase the solubility of iron after the formation of protein and iron complexes. Thus, protein–iron interaction was investigated at pH 7.0 with different protein : iron ratios (w/w), including 10 : 1, 20 : 1, 40 : 1 and 60 : 1. The protein amount was increased gradually to understand the effect of the protein content on iron chelation. After a 30 min interaction, the protein content significantly decreased in the complexes prepared at protein : iron ratios of 10 : 1 and 20 : 1, while the complexes prepared at protein : iron ratios of 40 : 1 and 60 : 1 maintained their soluble protein content during a 2 h incubation period (Fig. 2A). The sharp decrease in the protein content of complexes prepared at low protein : iron ratios (10 : 1 and 20 : 1) might be explained by the reduced protein solubility and detectability with the Bradford reagent due to protein aggregation mediated by an extensive intermolecular binding of iron among protein mole-

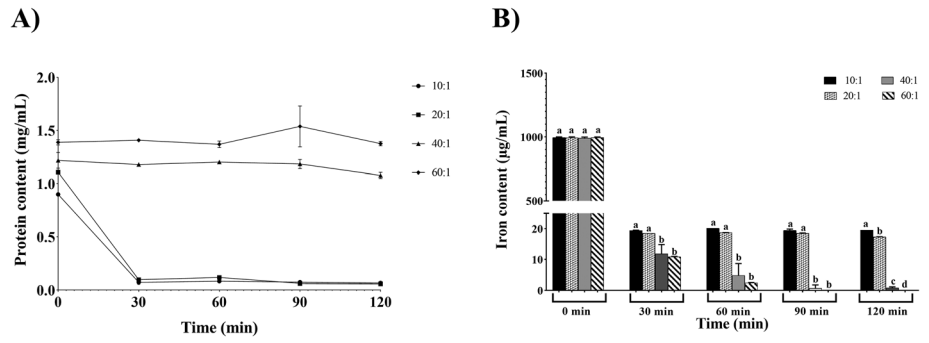

Fig. 2 Protein–iron binding profile at different ratios with respect to time. (A) Protein content and (B) iron content. The amounts are shown as protein : iron ratio (10 : 1 (10 mg : 1 mg in 1 mL), 20 : 1 (20 mg : 1 mg in 1 mL), 40 : 1 (40 mg : 1 mg in 1 mL), or 60 : 1 (60 mg : 1 mg in 1 mL)). Bars with lowercase letters are significantly different ($p < 0.05$). Results are shown as mean ± standard deviation.

cules. The intramolecular binding of iron molecules to reactive amino acid side chains of individual protein molecules could also reduce protein detectability by the Bradford reagent. Then, the Lowry assay was used as an alternative method to confirm Bradford assay data, and very similar results were observed (data not shown). As a reverse approach, we measured free iron levels in samples of protein–iron complexes (Fig. 2B). The incubation of protein–iron complexes for 30 min caused chelation of a minimum of 98% of the total free iron (from 1000 μg mL^{-1} to 20 μg mL^{-1}) in all protein–iron complexes. Residual free iron (1.8 to 2%) was maintained in protein–iron complexes in protein : iron ratios of 10 : 1 and 20 : 1 during a 2 h incubation period. However, iron binding and chelation occurred very extensively in protein : iron ratios of 40 : 1 and 60 : 1. Thus, the free soluble iron content in these solutions reduced to undetectable levels after 60 min of interaction. These results clearly showed that the majority of the iron binding by protein chelating groups is completed within 30 min and the iron binding capacity of the protein was correlated with an increased amount of protein. Lentil proteins reduce free iron levels at a neutral pH, which is similar to the pH of the lumen. The chelation strategy of free iron not only reduces free radical production but also increases iron bioavailability. However, the degree of protein–iron solubility and aggregation (degree of polymerization) should play a significant role in iron bioavailability. Our results also suggest that free iron salt was insoluble at the neutral pH at which the protein was soluble, suggesting that chelation of iron with protein could minimize the effects of pH on iron bioavailability.

2.2. Hydrolysis of protein–iron complexes

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 protein hydrolysation. The hydrolysation of protein–iron complexes leads to the production of peptide–iron complexes, which are a functional form of protein–iron complexes. Eckert *et al.* (2014)[7] revealed that barley protein hydrolysates and their purified fractions increased the solubility of different metal ions remarkably and concluded that higher solubility of metal ions at acidic pH (3–5) facilitated their absorption. Protein–iron interactions through peptides must survive the challenging gastrointestinal digestion conditions such as effects of various digestive enzymes and different pH fluctuations to exhibit a beneficial effect.[5] Before the *in vitro* enzymatic hydrolysis step, experimental conditions were determined by simulating the protocol (see the supplementary document for tables and figures). The experimental model was chosen from simulated data with conditions such as 8% pepsin (w/w, protein basis), 4% pancreatin (w/w, protein basis) with 2 h gastric and 2 h intestinal incubation, respectively. These parameters were experimentally validated and different protein : iron ratios were utilized in hydrolysis regarding the validated protocol. This information allowed us to figure out the optimal hydrolysis conditions for protein–iron complexes. The selection of hydrolysation protocol was critical for *in vitro* cell culture experiments to minimize the cofounding factors including minerals, bile acids, *etc*. We only intended to hydrolyse protein–iron complexes by pepsin and pancreatin, which is a very common method for protein hydrolysation.[15] Solubility and stability are the crucial factors for mineral bioavailability.[16] Therefore, the stability of protein–iron complexes was evaluated under optimized simulated *in vitro* hydrolysis conditions with different protein : iron ratios (w/w): 5 : 1, 10 : 1, 20 : 1, 40 : 1, and 60 : 1, respectively (Fig. 3). We observed that free iron was not detected in protein : iron solutions in the ratio 10 : 1 to 60 : 1 (Fig. 3A). We also included the 5 : 1 ratio in order to test whether all iron

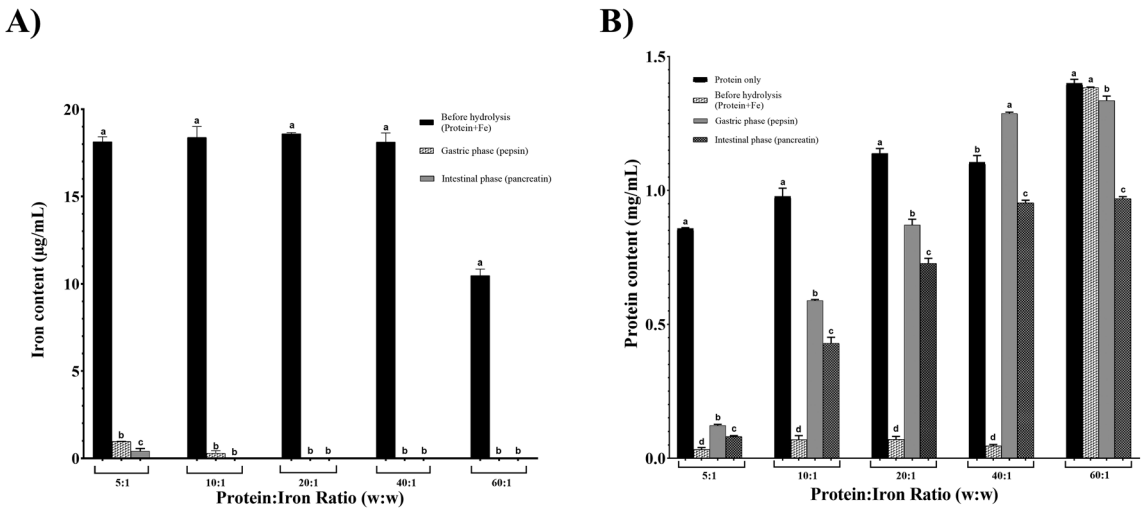

Fig. 3 The profile of (A) the free iron content (B) protein content of protein–iron complexes at different protein : iron ratios (w/w) after *in vitro* hydrolysis. Protein–iron was combined in the solution following pepsin incubation. Next, this hydrolysate was incubated with pancreatin. Protein and 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.

was bounded to protein in any ratio lower than 10 : 1. After hydrolysis, unbound free iron was observed in the 5 : 1 ratio samples, suggesting that the protein content was critical for the chelation of iron. Furthermore, when the protein extract was incubated with iron (without hydrolysation), unbound residual iron was observed in the solutions of all protein : iron ratios in time points in the range 0–60 min (Fig. 2B). However, after pepsin and pancreatin hydrolysis of protein–iron complexes obtained at ratios of 10 : 1 to 60 : 1, free unbound iron was not detected, indicating that hydrolysis enhanced iron binding capacity, most likely by the production of peptides. According to the literature, the sequential hydrolysis (first pepsin and second pancreatin) system causes the formation of a greater variety of peptide sequences with more cut-off sites that could act as functional metal-binding sites (Polanco-Lugo *et al.*, 2014; Sánchez-Chino *et al.*, 2018).[17,18] Miao *et al.* (2019)[19] also showed that pepsin and pancreatin hydrolyses are of great importance for iron chelation of casein hydrolysates in terms of exposure to more active sites for bivalent iron binding.

2.3. Effect of the hydrolysed protein–iron complex on iron dependent gene regulation

Body iron homeostasis is controlled by intestinal iron absorption because mammals do not have active excretion mechanisms.[10] Enterocyte cells are responsible for dietary nutrient absorption and constitute 95% of total intestinal cell types.[20] The physiology of human enterocyte cells has been modelled with very different cell lines. The human epithelial colorectal adenocarcinoma cell line, Caco-2, has been used to investigate iron metabolism since it is very responsive to iron deficiency anemia *in vitro*.[21–24] DFO treatment induced mRNA 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). Transcriptional regulation of those genes is very sensitive for treatments of the DFO and iron.[23] 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.[25] Furthermore, *ANKRD37* gene is regulated by HIF-2α protein in Caco-2 cells under DFO and Fe treatment. We observed that the DFO treatment of Caco-2 cells induced *DMT1*, *TFR*, and *ANKRD37* mRNA expression levels compared to the control group (Fig. 4A, B and C). We chose 10 : 1 and 60 : 1 protein : iron ratios, which did not have any free iron after hydrolysation of protein–iron complexes (or can be called peptide–iron complexes). The second criterion for the selection of those two ratios was to test the possible effects of low (10 : 1) and high (60 : 1) peptide–iron complexes on iron regulating gene mRNA expression. We observed that *DMT1*, *TFR*, 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 10 : 1 and beyond, indicating that hydrolysed protein–iron complexes reduced the anemic condition in these cells. However, the same significant reduction was not observed for the 60 : 1 protein : iron ratio except for the *ANKRD37* gene, which is very responsive to intracellular iron status. These differences in gene regulation might be explained in two ways. The hydrolyzation of 60 : 1 protein : iron complex might cause excessive production of free peptides. These free peptides might interfere with the absorption of the iron–peptide complex into cells or a high amount of peptides reduces the molecular effects of the iron into cells. However, these hypotheses must be tested by further experiments. Our results suggested that the physiological effect of peptide–iron complexes on iron regulating gene mRNA expression might depend on peptide concentration *in vitro*. Eckert *et al.* (2014)[7] pointed out that each specific barley derived peptide molecules might have different binding abilities towards a variety of metal ions. The peptide and mineral interactions have also been investigated in different protein sources, including chickpeas, fruit beverages, and barely, and it was concluded that peptides from these sources reduce the toxicity of iron and calcium minerals and also increase mineral bioavailability.[26] Moreover, the synthetic form of the heptapeptide enhanced iron bioavailability in Caco-2 cells, indicating that iron binds to the peptide in an amino acid specific manner.[27] The stability of the peptide–iron complex is crucial during digestion

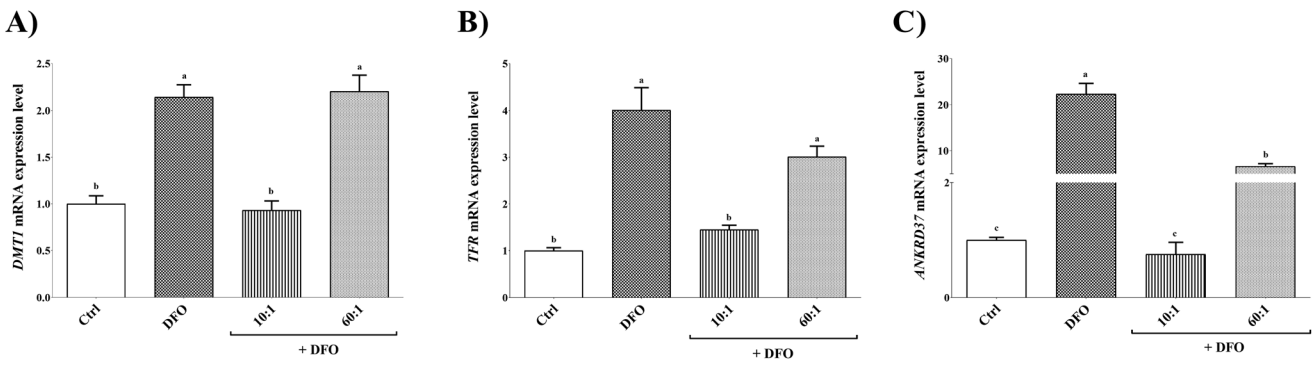

Fig. 4 The effect of hydrolysed peptide–iron complexes on iron metabolism and hypoxia-induced gene expression on anemic cells growing on 12-well inserts. (A) *DMT1*: Divalent metal transporter 1, (B) *TFR*: Transferrin receptor, (C) *ANKRD37*: Ankyrin repeat domain 37. Data were presented as mean ± standard deviation. Bars with different lowercase letters are significantly different ($p < 0.05$).

and absorption, since free iron causes toxicity in cells. It has been proposed that specific peptides increase iron absorption by increasing their solubility. To achieve this, proposed mechanisms are to insert peptides and iron into enterocyte cell in a paracellular way and/or *via* a peptide transporter.[5] Lin *et al.* (2015)[28] found that chelating hydrolysed proteins from hairtail fish species given to anemic rats increased hemoglobin and ferritin concentrations. It has also been shown that introducing iron–whey peptide complex into food products provides acceptable sensory features and reduces the risk of free iron toxicity. These observations suggest that peptides enhance iron bioavailability and that chelation of iron by peptides reduces the toxicity of free iron.

3. Conclusions

In this study, lentil protein extract was able to chelate iron, and hydrolysation of the protein–iron complexes enhanced the iron binding capacity of proteins. This suggests that lentil proteins might reduce free iron toxicity by chelating iron. Furthermore, hydrolysed protein (peptide)–iron complexes significantly reduced *DMT1*, *TFR*, 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 complex was 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 lentil 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.

4. Materials and methods

4.1. Materials

Red lentils were purchased from a supermarket in Izmir (Turkey) and refrigerated until use. The bovine serum albumin (CAS: 9048-46-8), ferrous sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$) (CAS: 7782-63-0), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (Ferrozine) (CAS: 69898-45-9), glacial acetic acid (CH_3CO_2H) (CAS: 64-19-7), hydrochloric acid (HCl) (CAS: 7647-01-0), sodium hydroxide (NaOH) (CAS: 1310-73-2), pepsin from porcine gastric mucosa (≥250 units per mg solid) (CAS: 9001-75-6), and pancreatin from porcine pancreas (8 × USP) (CAS: 8049-47-6) were purchased from Sigma-Aldrich (St Louis, MO, USA).

4.2. Protein extraction

The crude protein extract from lentils was prepared by the alkaline extraction method.[14] Briefly, 50 g of the lentils was rehydrated overnight in 500 mL of deionized water at 4 °C. The mixture was then homogenized by using a Waring blender for 2–4 min, its pH was adjusted to 9.5 with 1 mol L^{-1} NaOH, and it was extracted by magnetic stirring for 2 h at room temperature. The insoluble debris was then removed by filtering through cheesecloth, and the thinned slurry was used in protein purification applying classical isoelectric precipitation (IEP). The protein extracts of lentils were first clarified by centrifugation for 30 min at 15 000*g* at 4 °C. The proteins in the supernatant were then precipitated by the IEP by adjusting the pH of extracts to 4.5 with 1 mol L^{-1} acetic acid. The precipitated proteins were collected by centrifugation and 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 after suspending them in distilled water (Labconco, FreeZone, 6 L, Kansas City, MO, USA). The lyophilized lentil protein extracts were stored at −20 °C until they were used.

4.3. The total and water-soluble protein contents of protein extracts

The total nitrogenous compounds in protein extracts were determined by the Kjeldahl method using an automated testing machine (Gerhard vapodest 50s and Kjeldahl Therm, Germany). The total protein contents (TPrC) were calculated by using the conversion factor of 6.25. The water-soluble protein content (WSPC) of the extracts before and after hydrolysis was determined by the Bradford method.[29] The protein analysis of each sample was conducted with three replicates, and results were expressed as grams of soluble protein per one gram of protein extract (g g^{-1}).

4.4. Measurement of ferrous ion amount

The ferrous ion content of supernatants from the protein–iron complexes was determined according to the method of Ward and Legako (2017)[30] with minor modifications before and after the hydrolysis assay. Briefly, 100 μL of the protein extract was mixed with 250 μL of 1 mM ferrozine in a 96-well microplate. The solutions were then allowed to stand for 10 min at room temperature. After incubation, the absorbance was read at 562 nm with a microplate reader. The iron analysis of each sample was conducted with three replicates, and results were expressed as μg mL^{-1}.

4.5. Determination of iron solubility at various pH

To obtain the solubility profile of iron, $FeSO_4{\cdot}7H_2O$ was dispersed in water (1000 μg mL^{-1}) and the pH was adjusted with 1 mol L^{-1} NaOH or HCl to 2, 3, 4, 4.5, 5, 6, 7, or 8. The dispersions were kept at room temperature (25 ± 2 °C) under stirring (100 rpm) for 30 min, and they were centrifuged at 4500*g* for 20 min at 4 °C. The iron content in the supernatants was determined by the ferrozine assay as discussed previously in Section 4.4.

4.6. Formation of protein–iron complexes

The lentil protein extract was dispersed in deionized water, and the pH was adjusted to 7 with 0.1 mol L^{-1} NaOH. The dis-

persions were kept at room temperature under stirring (100 rpm) for 30 min. The same amounts of $FeSO_4 \cdot 7H_2O$ were then added into protein solutions to achieve protein : iron ratios of 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), or 60 : 1 (60 mg : 1 mg in 1 mL). The soluble protein contents and iron contents of the solutions were then monitored using the given procedures for 2 h by taking samples at 30 min intervals.

4.7. *In vitro* enzymatic hydrolysis of protein–iron complexes

An enzymatic hydrolysis protocol was simulated by a Box–Behnken design to estimate percentage of enzyme : protein ratio and gastric incubation time (see ESI† for detailed protocol). The protein–iron complexes were formed as previously explained in the section 4.6 and digested according to the assay described in González-Montoya *et al.* (2018)[15] with minor modifications based on the optimization study. Briefly, the protein–iron solutions that formed at the specified concentrations were maintained at pH 2.0 with 1 N HCl and then digested with pepsin (8% w/w, protein basis) at 37 °C for 2 h. After incubation, the solution was adjusted to pH 7.5 with 1 N NaOH and further digested with pancreatin (4% w/w, protein basis) at 37 °C for 2 h. The hydrolyzation of protein–iron complexes was stopped in an ice bath for 10 min. The digest was centrifuged at 4500*g* for 20 min at 4 °C. The supernatants were collected, and their free ferrous iron content was analysed immediately for further cell experiments.

4.8. Cell culture

Caco-2 cell line 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^{-1}) and, 1% nonessential amino acid solution (Gibco, Cat. No. 11140). They were kept 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 20th and 30th passages.

4.9. Induction of iron deficiency anemia and treatment of hydrolysed 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 per well into classical cell culture plates (12-well plates) (Costar, Cambridge, MA). Iron deficiency anemia was induced in the cells at the 10thday of seeding using a chemical agent (Deferoxamine, DFO) at a concentration of 200 μM. After incubation of DFO for 24 h, anemic cells were treated with protein–iron complexes (protein : iron ratio as 10 : 1 and 60 : 1 (10 mg : 1 mg and 60 mg : 1 mg in 1 mL, respectively) for a further 18 h in the incubator.

4.10. Total RNA isolation quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells with the RNAzol reagent (MRC, Cat. No.: RN190) following manufacturer's protocol. One microgram of each total RNA was converted to cDNA (Lifetech, Cat. No.: 4368814). qRT-PCR was performed on an ABI StepOnePlus instrument (Lifetech, CA, USA) by gene-specific oligonucleotide primers (ANKRD37: Forward – AGCAGTCGCCTGTCCACTTAGC, Reverse – AGCAGGCTTAGGCACTCCAGG; CypA: Forward – TACGGGTCCTGGCATCTTG, Reverse – CGAGTTGTCCACAGTCAGCA; DMT1: Forward – TGCATCTTGCTGAAGTATGTCACC, Reverse – CTCCACCATCAGCCACAGGAT; TFR: Forward – TCAGAGCGTCGGGATGATATCGG, Reverse – CTTGATCCATCATCATTCTGAACTGCC) and SYBR-Green mix (Lifetech, Cat. No.: 4367659). The human cyclophilin A (CypA) mRNA was used as a housekeeping gene to normalize data. Mean fold changes in gene-specific mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ analysis method.[31]

4.11. 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. Design Expert version 11 was used for the statistical experimental design for all the *in vitro* enzymatic hydrolysis experiments with the response as soluble protein content (grams of soluble protein per gram of protein extract). The results were considered statistically significant for P values less than 0.05.

Abbreviations

Caco-2	Human epithelial colorectal adenocarcinoma cell line
mRNA	Messenger ribonucleic acid
DMT1	Divalent metal transporter-1
TFR	Transferrin receptor
ANKRD37	Ankyrin repeat domain 37
IDA	Iron deficiency anemia
IEP	Isoelectric precipitation
DFO	Deferoxamine

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Conflicts of interest

The authors declare that there are no conflicts to declare.

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E. E. and S. G. designed the investigation, wrote the manuscript, and prepared figures; and E. E. performed the experiments. We thank Dr Ahmet Yemenicioğlu, who provided technical suggestions for protein isolation, edited manuscript, and discussed the data. All authors approved the final manuscript. The authors are also grateful to the Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center for providing their laboratory facilities.

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