JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 4593–4597 4593
DOI:10.1021/jf9000204

Identification and Characteristics of Iron-Chelating Peptides from Soybean Protein Hydrolysates Using IMAC-Fe^{3+}

Ying Lv,[†] Qi Liu,[†] Xiaolan Bao,[‡] Wuxia Tang,[§] Baichong Yang,[†] and Shuntang Guo*,[†]

[†]College of Food Science & Nutritional Engineering, China Agricultural University, 17 Qinghua Donglu, Haidian District, Beijing 100083, China, [‡]Department of Food Science, Inner Mongolia Agricultural University, 306 Zhaowuda street, Huhehaote City 010018, China, and [§]Department of Food Science, Sichuan Agricultural University, 46 Xinkang street, Ya'an City 625014, China

The iron-chelating peptides from soybean protein hydrolysates (SPH) were investigated using immobilized metal affinity chromatography (IMAC). The results demonstrated that SPH could absorb on the IMAC-Fe^{3+} column, while the capability of the binding iron was different in SPH (10−30 kDa), SPH (3−10 kDa), and SPH (1−3 kDa). The highest binding amount on the column occurred with SPH (10−30 kDa). With the IMAC method, the iron-chelating peptides were shown to be formed at pH lower than 5.5, and they were not affected by NaCL with the concentration between 0.1 mol/L and 1 mol/L, while the iron-chelating peptides could be partially disrupted by 0.02 mol/L Na_2HPO_4 at pH 8.0. Furthermore, the iron-chelating peptides were identified with reversed phase (RP)-HPLC, SDS−PAGE, and MALDI-TOF MS/MS. The binding characteristics of the SPH on IMAC-Fe^{3+} and the sequences of the iron-chelating peptides revealed that binding sites between SPH and iron might be the carboxyl groups of Glu and Asp residues.

KEYWORDS: Purification; iron-chelating peptides; soybean protein hydrolysates; immobilized metal affinity chromatography

INTRODUCTION

Iron is an important micromineral in the human body, and its deficiency is responsible for many diseases (*1*). Various dietary components influence iron absorption. Many animal tissues enhance iron absorption for the special amino acids of the peptides during proteolytic digestion, such as His, Glu, Asp, and Cys (*2*−*4*). These amino acids can form soluble complexes with iron and enhance iron absorption. While there have been conflicting results in plant protein, soybean protein acts prominently as an iron absorption inhibitor (*5*). However, it has been reported that soybeans appeared to be a good source of nutritional iron in women with low iron stores (*6*). Macfarlane et al. (*7*) investigated the effect of a variety of traditional oriental unfermented and fermented soy products on iron absorption in 242 Indian women. They found that iron absorption was significantly improved and that it may be related to the protein composition of the products. In connection with this, Japanese soy sauce was reported to promote iron absorption (*8*), and a relatively high iron bioavailability also was observed from consumption of iron fortified soy sauce, which may be due to the presence of fermentation products (*9*). In summary, these studies have indicated that soybean peptides might be related to iron absorption.

Enzymatic hydrolysis of food protein can release peptides that are able to chelate with metal and show different biological activities. For example, peptides hydrolyzed from casein have been shown to bind and increase calcium absorption in the intestine (*10*), and they can also enhance iron uptake by Caco-2 cells (*11*). Chickpea protein hydrolysates chelating with copper possess antioxidative properties (*12*). Our previous work reported that soybean protein hydrolysates (SPH) could bind with calcium forming soluble SPH-calcium complex (*13*) and promote calcium uptake by Caco-2 cells (*14*).

In this work, we describe the interaction between SPH and iron using iron−chelated solid support. Immobilized metal affinity chromatography (IMAC), proposed by Porath et al. (*15*) in 1975, is a selective tool to separate metal-binding peptides because of the accessible His, Ser, Cys, Glu, and Asp residues on the surface of the peptides (*2*−*4*). The effect of peptide binding with immobilized metal ions is related to pH value (*16*), salt concentration (*17*, *18*), and elution buffer (*19*). Our aim was to find suitable conditions for selective separation of SPH using IMAC-Fe^{3+}, investigate the interaction between SPH and iron, purify the iron-chelating peptides, and identify the structural characteristics of the peptides.

MATERIALS AND METHODS

Materials. The study utilized defatted soybean flakes provided by Yihai Liangyou Co. Ltd. (Qinhuangdao, China). Protease M and deamidase were purchased from Amano Enzyme Co. Ltd. (Nagoya, Japan). Different molecular weight ultrafiltration membranes were products of Zhongke Membrane Technology Co. Ltd. (Beijing, China). Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden). Iminodiacetic acid

*Corresponding author. Tel/Fax: +86-10-62737634. E-mail: shuntang@cau.edu.cn.

 Published on Web 05/14/2009

(IDA) was purchased from Sigma (USA). IDA-Sepharose 6B was prepared according to the method of Porath et al. (*20*). All other chemicals were of analytical reagent grade.

Sample Preparation. Soybean protein isolates (SPIs) were extracted as described by Sorgentini et al. (*21*) with some modifications. SPIs were prepared from defatted soybean flakes by extraction for 1.5 h at room temperature with water adjusted at pH 8.0 with 2 mol/L NaOH (water/flour = 10:l) and then centrifuged at 3,000*g* for 20 min. After that, the supernatant was adjusted to pH 4.5 with 2 mol/L HC1, kept for 0.5 h at room temperature, and then centrifuged (3,000*g*, 20 min). The resultant pellet was washed with water twice to remove the soluble residues at pH 4.5, and then water was added. This dispersion was named SPI. In order to prepare SPH, the pH of the SPI solution was adjusted to 3.0 and denatured for 10 min in a boiling water bath, and then protease M was added (protein content of 20 g/L, E:S = 1:100). Enzymatic hydrolyses were then performed on these solutions at 50 °C for 60 min. Following digestion, the solutions were heated in a boiling water bath for 5 min to deactivate protease M and afterward cooled to room temperature and neutralized to pH 7.4. Subsequently, the solutions were centrifuged at 3,000*g* for 20 min, and deamidase was then added to the resulting supernatants (E:S = 1:50), which were then hydrolyzed at pH 7.0 in 50 °C for 180 min. After hydrolysis, the solutions were heated in a boiling water bath for 5 min to deactivate deamidase. Then they were cooled to room temperature and centrifuged (3,000*g*, 20 min). The resultant supernatants were collected as SPH. This solution was ultrafiltrated with membranes of molecular weight cut offs (MWCO) of 1, 3, 10, and 30 kDa at 4 °C. Three fractions collected as SPH (1−3 kDa), SPH (3−10 kDa), and SPH (10−30 kDa) were lyophylized.

Chromatographic Procedures. The column was packed with IDA-Sepharose 6B (25 mL) and then incubated with 0.2 mol/L $FeCl_3$ (40 mL) for iron charging. After the column was washed with distilled water for 8−9 bed volumes to remove the unbound iron, the nonspecific bound iron was removed by washing the column with 0.05 mol/L acetic acid (HAC) buffer at pH 4.0 for 5−6 bed volumes. The column was equilibrated with different equilibrating buffers. Then, 1 mL of SPH solution (20 mg/mL) was loaded onto the column. The wash volume was collected and designated the nonadsorbed fraction. The bound peptides were afterward eluted with the appropriate elution buffers. The absorbance of the eluate was monitored at 210 nm, and the flow rate was 1 mL/min. Regeneration of the column was achieved with 0.05 mol/L EDTA. All chromatographic experiments were carried out at room temperature.

Equilibrating Buffers with Different pH Values. Considering the effect of the pH values on the behavior of SPH on the affinity column, the buffers used for the peptide adsorption experiment were as follows: (a) 0.05 mol/L sodium acetate−acetic acid (NaAC-HAC) buffer at pH 5.0; (b) 0.05 mol/L NaAC-HAC buffer at pH 5.5; (c) 0.05 mol/L morpholinoethane sulfonic acid (MES), adjusted to pH 6.0 with NaOH; (d) 0.05 mol/L MES, adjusted to pH 6.5 with NaOH. All of these buffers contained 0.1 mol/L NaCl.

Equilibrating Buffers with Different Salt Concentrations. Considering the effect of the salt concentration on the behavior of SPH on the affinity column, the buffers used for the peptide absorption experiment were as follows: 0.05 mol/L NaAC-HAC buffer at pH 5.5, containing (e) without NaCl; (f) 0.1 mol/L NaCl; (g) 0.5 mol/L NaCl; and (h) 1 mol/L NaCl.

Elution Buffers. The elution buffers used in these experiments were as follows: (i) 0.02 mol/L Na_2HPO_4; (j) 0.02 mol/L NaH_2PO_4; (k) 0.02 mol/L NH_4Cl; (l) 0.02 mol/L imidazole; (m) 0.02 mol/L sodium glutamate; (n) 0.02 mol/L malonic acid.

Identification of Iron-Chelating Peptides. The fractions collected from the IMAC-Fe^{3+} column were applied to reversed-phase (RP)-HPLC on a protein and peptide Zorbax SB-C18 column (9.4 × 150 mm, 5 μm) from Agilent Technologies. The column was equilibrated using water with 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. The linear gradient of 80% acetonitrile (ACN) (in 0.1% TFA) was applied from 0 to 100%. Elution was monitored at 214 nm. The collected fractions were lyophylized. Then, the separated SPH was analyzed with sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the bands on the gel were cut to analyze with the matrix-assisted laser desorption/ionization time-of-flight mass spectrometer/mass spectrometer (MALDI-TOF MS/MS; TraflexIII TOF/TOF, Bruker, Germany).

Determination of the Concentration of Peptide. Concentration of the peptide was determined by the method of Lowry et al. (*22*). Bovine serum albumin (BSA) was used as a reference protein.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was carried out on a vertical slab gel with a thickness of 1 mm (BIO CRAFT MODEL BE-210N, Japan) using an alkaline discontinuous buffer (*23*). The ratio of acrylamide and bisacrylamide were 30:1. The concentrations of the stacking gel and the separating gel were 4% and 12.5%, respectively. The protein bands were stained with Coomassie brilliant blue G-250 in the gel and scanned using an HP scan instrument (HP 1000, U.S.A.).

Statistical Analysis. The values are reported as the means ± SD, $n = 2$. ANOVA was used to determine the significance of differences of the data. $P < 0.05$ was considered as significant. Statistical analysis was performed using the OriginPro 7.5 software package (OriginLab Corp., MA, U.S.A.).

RESULTS AND DISCUSSION

Usually, soybean protein has been reported to inhibit iron absorption not only for phytate acid but also the protein itself (*24*). However, Macfarlane et al. (*7*) found that iron absorption in Indian women was improved after ingesting traditional fermented soy products. Fermentation results in a modification of the soybean protein structure (*25*), and the peptides (> 12 kDa) were shown an inverse relationship with iron absorption (*24*). It seems that the molecular weight of the peptides were associated with iron bioavailability. In this study, SPH with different molecular weights prepared by ultrafiltration were used to study their iron-binding capacity. As shown in **Table 1**, SPH can adsorb on the IMAC-Fe^{3+} column, and under the same conditions, SPH (10−30 kDa) possesses more affinity with IMAC. The unadsorbed peptides were 41.45%, which was significantly lower than that of SPH (3−10 kDa) and SPH (1−3 kDa) ($p < 0.01$). Hence, SPH (10−30 kDa) was selected for further purification.

To identify the iron-chelating SPH and reveal the interaction between SPH and iron, IMAC-Fe^{3+} was used to separate the peptides, and at the same time the effect of pH value, salt concentration, and elution reagent was also investigated.

The effect of pH on the binding capacity of SPH is shown in **Table 2**. At pH 5.0 and pH 5.5, the nonadsorbed SPH was almost the same as 41.45%. With the increase of pH, the unadsorbed SPH increased, and the affinity of SPH on IMAC decreased. This result is in agreement with the work of Zachariou et al. (*19*), who found that protein binding to the immobilized Fe^{3+} adsorbent was pH dependent. Usually, Glu, Asp, Ser, and His residues of protein can bind with iron. The iron prefers to chelate with the oxygen-rich group, such as the oxygen of the phosphate group and the carboxyl groups of Asp and Glu, and the nitrogen-rich group of the imidazole group of His (*19*). As we know, the pI of Asp and Glu is 2.77 and 3.2, respectively, and in the presence of a pH 5.5 solution, the carboxyl groups of Asp and Glu would

Table 1. Nonadsorbed SPH with Different Molecular Weights on IMAC-Fe^{3+} (Means ± SD, $p < 0.01$, $n = 2$)[a]

	SPH (10−30 kDa)	SPH (3−10 kDa)	SPH (1−3 kDa)
nonadsorbed SPH (mg)	8.285 ± 0.001 a	11.13 ± 0.12 b	12.42 ± 0.09 b

[a] The different letters indicate statistically significant differences.

Table 2. Nonadsorbed SPH (10−30 kDa) on IMAC-Fe^{3+} at Different pH Values (Means ± SD, $p < 0.01$, $n = 2$)[a]

	pH 5.0	pH 5.5	pH 6.0	pH 6.5
nonadsorbed SPH (10−30 kDa) (mg)	8.290 ± 0.007 a	8.285 ± 0.001 a	9.805 ± 0.014 b	10.975 ± 0.068 c

[a] The different letters indicate statistically significant differences.

Table 3. Nonadsorbed SPH (10−30 kDa) on IMAC-Fe^{3+} at Different Concentrations of NaCl (Means ± SD, $p < 0.01$, $n = 2$)[a]

	concentration of NaCl (mol/L)				
	0	0.05	0.1	0.5	1.0
nonadsorbed SPH (10 −30 kDa) (mg)	4.980 ± 0.072 a	7.650 ± 0.013 b	8.285 ± 0.001 c	8.090 ± 0.003 c	8.160 ± 0.013 c

[a] The different letters indicate statistically significant differences.

Table 4. Eluted SPH (10−30 kDa) from IMAC-Fe^{3+} with Different Elution Buffers (Means ± SD, $p < 0.01$, $n = 2$, ND = Not Detected)[a]

	elution buffer (0.02 mol/L)				
	Na_2HPO_4	NaH_2PO_4	imidazole	sodium glutamate	NH_4Cl
eluted SPH (10−30 kDa) (mg)	8.190 ± 0.016 a	5.975 ± 0.011 b	2.450 ± 0.051 c	0.685 ± 0.001 d	ND

[a] The different letters indicate statistically significant differences.

ionize and chelate with iron, while above pH 6.0, the hydroxide ions would compete with carboxyl groups to bind with iron. Hence, the binding capacity of SPH decreased with the rise of pH. Meanwhile, the pI of His is 7.6, and it would not ionize to bind with iron at pH 5.5. From those results, it seemed that the binding sites might be the carboxyl groups of Glu and Asp but not the His. SPH contains about 20% Asp and Glu (*13*), which might provide the carboxyl groups to bind with iron.

Salt concentration of the equilibrating buffer plays an important role in the binding capacity of the affinity column. The effect of ionic strength in the balance buffer on binding capacity of SPH to the IMAC-Fe^{3+} column was investigated with the buffers containing different NaCl concentration from 0 to 1.0 mol/L. The results are shown in **Table 3**. When the NaCl concentration increased from 0 to 0.1 mol/L, the binding amount of the SPH decreased. However, the amount of binding SPH did not change when the NaCl concentration was higher than 0.1 mol/L. This result indicated that the electrostatic interaction and specific interaction (coordinate interaction) between SPH and iron promoted the adsorption of SPH on the IMAC-Fe^{3+} column. When the ionic strength in the balance buffer increased, the nonspecific interaction was reduced. However, the specific interaction could not be disrupted by NaCl. This result is consistent with what Chen et al. (*17*) and Lin et al. (*18*) have reported, on the existence of a manifold binding mechanism involving hydrophobic, electrostatic interaction, and specific interaction of solute with immobilized ions.

In order to selectively separate the SPH from the column, different elution reagents were explored. The results are shown in **Table 4**. The amount of eluted SPH (10−30 kDa) from the IMAC-Fe^{3+} column decreased as follows: Na_2HPO_4 > NaH_2PO_4 > imidazole > sodium > glutamate > NH_4Cl. The malonic acid stripped most of the iron from the column and could not be used to elute the SPH. The SPH recovered by Na_2HPO_4 was 40%, while imidazole and NH_4Cl, which eluted mainly His-chelated protein, could elute little SPH. This suggested that at the same basic conditions, PO_4^{3-} can reduce the interaction between SPH and iron, which further indicated that the binding sites might mainly be the carboxyl groups.

According to the above results, 0.05 mol/L, NaAC-HAC buffer at pH 5.5, containing 0.1 mol/L NaCl, was selected as the equilibrating buffer and 0.02 mol/L Na_2HPO_4 as the elution buffer. The unadsorbed fraction was eluted with the equilibrating buffer as peak 1. After that, when a gradient of 0−0.02 mol/L Na_2HPO_4 was applied, the SPH bound to the column was eluted

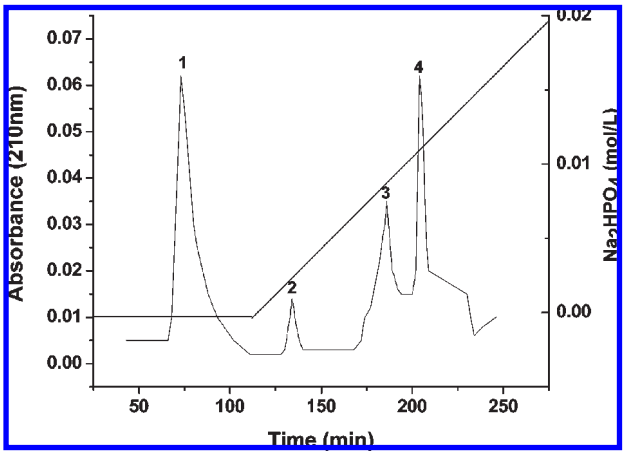

Figure 1. Isolation of SPH (10−30 kDa) with an IMAC-Fe^{3+} column.

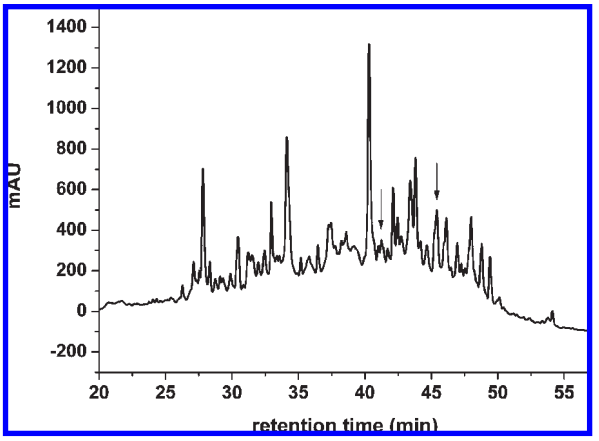

Figure 2. RP-HPLC profile of peak 3, which was eluted from an IMAC-Fe^{3+} column.

in three major peaks, 2, 3, and 4 (**Figure 1**). During this step, when the concentration of Na_2HPO_4 increased to 0.02 mol/L, the eluted peaks 3 and 4 were light yellow. At pH > 7.0, hydroxide ions also bind with iron to form the highly insoluble $Fe(OH)_3$, which might be the reason for the color. Fruthermore, the formation of $Fe(OH)_3$ also results in the disruption of peptide−iron complexes.

IMAC is a suitable method to separate metal-binding peptides. Lund et al. (*26*) selectively purified water-soluble phosphopeptides from cheese using IMAC-Fe^{3+} and got peptides with different phosphorylated serine residues. Also, Storcksdieck et al. (*2*) separated low molecular weight iron-binding peptides from muscle tissue using IMAC. Here, the peptides in peak 3, which were bound with IMAC-Fe^{3+}, were isolated for further purification.

Figure 2 shows the RP-HPLC profile of the fraction collected from peak 3. The peptides with different retention times (RTs) were separated using a 0.1% TFA−acetonitrile system. From those peptides, the peptides with RTs of 41 min and 45 min were collected for SDS−PAGE analysis. **Figure 3** shows the molecular weight of the peptides in the two peaks, and they were mainly the peptides >10 kDa (calculated according to SDS−PAGE with marker). The bands (arrows) were cut and analyzed with MALDI-TOF MS/MS. The sequences are shown in **Table 5**. There is a high content of Glu and Asp residues, and part of the structure lines as Asp-Glu-Asp and Glu-Glu-Glu, which might be crucial for binding with iron. Furthermore, the occurrence of those peptides and their sequences are reasonable considering the enzyme treatments. The enzymes used here were protease M and

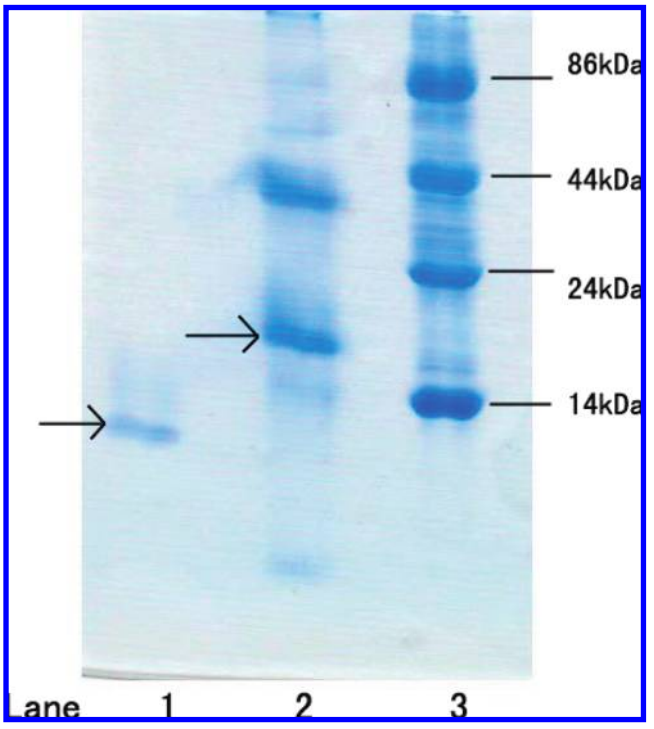

Figure 3. SDS−PAGE patterns of the peaks with different RT from RP-HPLC. Bands (arrows) were cut and analyzed with MALDI-TOF MS/MS. Lane 1, peptides with RT 41 min; lane 2, peptides with RT 45 min; lane 3: marker.

the deamidase, and the type of bonds that the protease M hydrolyzes is Gln, Ser, Met, and Cys residues. The first sequence was cut by the enzyme hydrolyzing the bond in Met from the 2S albumin subunit (**Table 5**).

Recently, consumption of fermented soy sauce with fortified iron was shown to produce a relatively high iron absorption in humans (*8*). The changes of soybean protein structure result from fermentation. The high molecular weight protein is hydrolyzed to the low molecular weight peptides, and thus, the iron bound by soybean protein might be easily released. That may be one reason that the fermented soybean products promote iron absorption (*7*−*9*). Interestingly, the soy sauce cannot overcome the strong inhibition of iron absorption from soy flour, while the iron absorption in the rice meal added with soy sauce was significantly improved (*9*). The fortified iron in the soy sauce might compete with the intact soybean protein in the soy flour, and this binding of intact soybean protein might be too tight to make the iron unavailable. That may also be the reason for the inverse relationship between the peptides (> 12 kDa) and iron absorption in the study of Indian women (*7*). In our study, the higher molecular weight of SPH was shown to account for more binding iron. Usually, it seems that the more iron one substance can bind, the more it can take to the intestine for absorption. However, too high molecular weight of soybean protein inhibits iron absorption. Thus, there must be soybean peptides with suitable molecular weight, which can not only bind enough iron but also release the iron easily. More research is needed on the effect of molecular weight on iron absorption.

Another reason for the higher iron bioavailability caused by fermented soybean products might be the peptides themselves. In some studies, the protein or peptides were reported as the meat factor, which promotes the nonheme iron absorption in humans (*2*, *3*, *27*). The iron-binding peptides from meat hydrolysates were separated, and the characteristics of those peptides are high content of Glu and Asp (*2*). Similarly, the His content of beef

Table 5. Amino Acid Sequence of Isolated Iron-Chelating Peptides from SPH (10−30 kDa)

RT (min)	molecular weight	detected sequence	complete sequence
41	11 kDa	LMNLAIRCRLGPMIG CDLSSDD	48 EKI QAGRRGEDGS DEDHILIRTM PGRINYIRKK EGKEEEEEGH MQKCCSEMSE LKSPICQCKA LQKIMDNQSE QLEGKEKKQM ERE**LMNLAIR CRLGPMIGCD LSSDD** 155 2S albumin 1 precursor [*Glycine max*]
45	17 kDa	DNQSEQLEGKEKK	1 MTKLTILLIA LLFIAHTCCA SKWQQHQQES CREQLKGINL NPCEHIMEKI QAGRRGEDGS DEDHILIRTM PGRINYIRKK EGKEEEEEGH MQKCCSEMSE LKSPICQCKA LQKIM**DNQSEQLEGKEKK**QM ERELMNLAIR CRLGPMIGCD LSSDD 155 2S albumin 1 precursor [*Glycine max*]

hydrolysates was also isolated and found to promote nonheme iron uptake in vitro (*3*). Furthermore, the nonheme iron absorption in humans was reported to be enhanced by meat protein fractions (*27*). From those studies, the peptides or protein with His, Glu, and Asp residues might play a crucial role in iron bioavailability. Thus, it seems that the amino acid composition and the sequence of the peptides determine their effects on iron absorption. In this study, the interactions between SPH and iron showed the conditions for the formation of iron-chelating peptides. According to the meat factor study, the iron-chelating peptides with high content of Glu and Asp residues might promote iron absorption, which could be the reason why some of the traditional oriental fermented soy products have a promotive effect on iron absorption (*7*−*9*). However, to understand whether the iron-chelating peptides with Glu and Asp residues can promote iron absorption, additional in vivo and in vitro studies are needed to confirm the findings reported here.

LITERATURE CITED

(1) Beard, J. L. Iron biology in immune function, muscle metabolism and neuronal functioning. *J. Nutr.* **2001**, *131*, 568s–580s.

(2) Storcksdieck, S.; Bonsmann, G.; Hurrell, R. F. Iron-binding properties, amino acid composition, and structure of muscle tissue peptides from in vitro digestion of different meat sources. *J. Food Sci.* **2007**, *72*, 19s–29s.

(3) Swain, J. H.; Tabatabai, L. B.; Reddy, M. B. Histidine content of low-molecular-weight beef proteins influences nonheme iron bioavailability in Caco-2 cells. *J. Nutr.* **2002**, *132*, 245–251.

(4) Taylor, P. G.; Martinez-Torres, C.; Romano, E. L.; Layrisse, M. The effect of cysteine-containing peptides released during meat digestion on iron absorption in humans. *Am. J. Clin. Nutr.* **1986**, *43*, 68–71.

(5) Schricker, B. R.; Miller, D. D.; Campen, D. V. Effects of iron status and soy protein on iron absorption by rats. *J. Nutr.* **1983**, *113*, 996–1001.

(6) Murray-Kolb, L. E.; Welch, R.; Theil, E. C.; Beard, J. L. Women with low iron stores absorb iron from soybeans. *Am. J. Clin. Nutr.* **2003**, *77*, 180–184.

(7) Macfarlane, B. J.; Riet, W. B.; van der; Bothwell, T. H.; Baynes, R. D.; Siegenberg, D.; Schmidt, U.; Tal, A.; Taylor, J. R.; Mayet, F. Effect of traditional oriental soy products on iron absorption. *Am. J. Clin. Nutr.* **1990**, *51*, 873–880.

(8) Baynes, R. D.; Macfarlane, B. J.; Bothwell, T. H.; Siegenberg, D.; Bezwoda, W. R.; Schmidt, U.; Lamparelli, R. D.; Mayet, F.; Macphail, A. P. The promitive effect of soy sauce on iron absorption in human subjects. *Eur. J. Clin. Nutr.* **1990**, *44*, 419–424.

(9) Fidler, M. C.; Davidsson, L.; Walczyk, T.; Hurrell, R. F. Iron absorption from fish sauce and soy sauce fortified with sodium iron EDTA. *Am. J. Clin. Nutr.* **2003**, *78*, 274–278.

(10) Sato, R.; Noguchi, T.; Naito, H. Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *J. Nutr. Sci. Vitaminol*. **1986**, *32*, 67–76.
(11) Argyri, K.; Miller, D. D.; Glahn, R. P.; Zhu, L.; Kapsokefalou, M. Peptides isolated from in vitro digests of milk enhance iron uptake by Caco-2 cells. *J. Agric. Food Chem*. **2007**, *55*, 10221–10225.
(12) Megías, C.; Pedroche, J.; Yust, M. M.; Girón-calle, J.; Alaiz, M.; Millán, F.; Vioque, J. Affinity purification of copper chelating peptides from chickpea protein hydrolysates. *J. Agric. Food Chem*. **2007**, *55*, 3349–3954.
(13) Bao, X. L.; Lv, Y.; Yang, B. C.; Ren, C. G.; Guo, S. T. A study of the soluble complexes formed during calcium binding by soybean protein hydrolysates. *J. Food Sci*. **2008**, *73*, 117–122.
(14) Lv, Y.; Bao, X. L.; Yang, B. C.; Ren, C. G.; Guo, S. T. Effect of soluble soybean protein hydrolysate-calcium complexes on calcium uptake by Caco-2 cells. *J. Food Sci*. **2008**, *73*, 2097–2103.
(15) Porath, J.; Carlsson, J.; Olsson, I.; Belfrage, G. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* **1975**, *258*, 598–599.
(16) Lin, F. Y.; Chen, W. Y.; Huang, S. Y. Selective separation of caseinphosphopeptides through immobilized metal ion affinity chromatography: effects of pH values, salt concentrations and degree of phosphorylation. *Bioprocess Eng*. **2000**, *23*, 467–471.
(17) Chen, W. Y.; Wu, C. F.; Liu, C. C. Interactions of imidazole and proteins with immobilized Cu (II) ions: effects of structure, salt concentration, and pH in affinity and binding capacity. *J. Colloid Interface Sci*. **1996**, *180*, 135–143.
(18) Lin, F. Y.; Chen, W. Y.; Sang, L. C. Microcalorimetric studies of the interactions of lysozyme with immobilized metal ions: effect of ion, pH value, and salt concentration. *J. Colloid Interface Sci*. **1999**, *214*, 373–379.
(19) Zachariou, M.; Hearn, T. W. M. Protein selectivity in immobilized metal affinity chromatography based on the surface accessibility of aspartic and glutamic acid residues. *J. Protein Chem*. **1995**, *14*, 419–430.
(20) Porath, J.; Olin, B. Immobilized metal ion affinity adsorption and immobilized metal ion affinity chromatography of biomaterials: Serum protein affinities for gel-immobilized iron and nickel ions. *Biochemistry* **1983**, *22*, 1621–1630.
(21) Sorgentini, D. A.; Wagner, J. R.; Añón, M. C. Effects of thermal treatment of soy Protein isolate on the characteristics and structure-function relationship of soluble and insoluble fractions. *J. Agric. Food Chem*. **1995**, *43*, 2471–2479.
(22) Lowry, O. H; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem*. **1951**, *193*, 265–275.
(23) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(24) Lynch, S. R.; Dassenko, S. A.; Cook, J. D.; Juillerat, M. A.; Furrell, R. F. Inhibitory effect of a soybean-protein-related moiety on iron absorption in humans. *Am. J. Clin. Nutr*. **1994**, *60*, 567–572.
(25) Fukushima, D. Soy proteins for food centering around soy sauce and tofu. *J. Am. Oil Chem. Soc*. **1981**, *58*, 346–354.
(26) Lund, M; Ardö, Y. Purification and identification of water-soluble phosphopeptides from cheese using Fe (III) affinity chromatography and mass spectrometry. *J. Agric. Food Chem*. **2004**, *52*, 6616–6622.
(27) Hurrell, R. F.; Reddy, M. B.; Juillerat, M.; Cook, J. D. Meat protein fractions enhance nonheme iron absorption in humans. *J. Nutr*. **2006**, *136*, 2808–2812.

Received January 3, 2009. Revised manuscript received April 21, 2009.