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Effects of Chemical Modification and Molecular Weight Distribution on Iron Binding Ability of Phytate-Removal Soybean Protein Isolate Hydrolysate

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Abstract: The aim of this study was focused on investigating the effects of chemical modifications and molecular weight distribution of phytate-removal Soybean Protein Isolate (SPI) hydrolysate on Degree of Hydrolysis (DH) and the iron binding ability. The chemical modifications included acid treatments, succinylation, acetylation and phosphorylation. Also the effects of molecular weight distribution of phosphorylated SPI trypsin hydrolysate on iron binding ability were studied. The results showed that acetic acid treatment can increase significantly the DH of SPI hydrolysate, but did not increase the iron-binding ability of SPI hydrolysate. Phosphorylation by Sodium Trimetaphosphate (STMP) can increase significantly the iron-binding ability, but did not change the DH. The STMP phosphorylation reaction time of 20 min was sufficient for improving the iron binding capacity and alkaline conditions were beneficial of phosphorylation reaction. The fraction of molecular size among 3 to 5 kDa exhibited highest iron binding ability, but there were no significant different in amino acid compositions between the four ultrafilteration fractions.

Key words: Amino acid composition, hydrolysis, mineral binding ability, phosphopeptides, phosphorylation, trypsin

INTRODUCTION

Soybean is one of the richest sources of natural protein among the plant. Good quality and functionality of its proteins, hypoallergenicity, low cost and surplus availability are other added features that motivated the researchers to explore its utility in a wide range of foods including the complementary foods. However, the presence of phytate at different levels in soybean based products has been shown to inhibit trace mineral absorption (Davidson *et al*., 2004). Phytate is known to have a strong affinity for zinc, iron and other trace minerals in foods and feeds. Its association with storage proteins results in relatively high concentrations of phytate in commercial soy protein products, such as soy meal, that are mainly used for swine and poultry feeds (Erdman, 1979). Phytate causes poor absorption of essential electrolytes and minerals and binds to proteins and co-precipitates with isoelectric soybean protein isolate (SPI) (Nicolas and Lawrence, 2007). Iron absorption increased four to five-fold when phytate was reduced from its native amount of 4.9-8.4 to less than 0.01 mg/g of isolate (Richard *et al*., 1992).

Soybean protein hydrolysates are physiologically better than intact proteins because their intestinal absorption appears to be more effective due to the increase in solubility and peptide content (Ziegler *et al*., 1998), thus making them attractive as a source of amino acids or peptides in human nutrition. Soybean protein hydrolysates have been shown to have various physiological activities, including hypolipidaemic and hypocholes-terolaemic properties (Yasuyuki and Yoshikawa, 2000), improvement in arterial compliance and endothelial function (Ringseis *et al*., 2005), insulin resistance and weight loss in obesity (Hermansen *et al*., 2001).

Chemical modifications resulted in increase significantly in solubility of the proteins (Zhang *et al.*, 2007), the small peptide content with desired antioxidant activity, the nitrogen soluble index of wheat gluten (Liao *et al*., 2010) and the amount of calcium bound to the soybean globulins (Kumagai *et al*., 2002). A limited trypsin hydrolysate of the isolated phosphopeptides exhibited an enhanced calcium binding ability (Yang *et al*., 2004). As hydrolysis proceeded, significant increases in surface hydrophobicity, protein solubility, emulsifying activity index and emulsion stability index were observed in the hydrolysates (Wu *et al*., 1998). Jiang and Mine (2001) found that the smaller fragment of less than 1 kDa and O-phospho-L-serine did not bind calcium to any significant extend, while phosphopeptide of 1-3 kDa showed a higher ability than casein phosphopeptides to render soluble calcium. The research of the iron-chelating peptides from soybean protein hydrolysates demonstrated that the highest binding amount on the column occurred with 10-30 kDa hydrolysate, while the capability of the binding iron was different in the three molecular size fractions (Lv *et al*., 2009).

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However, few works have been published focusing on the effect of physical and chemical modifications before hydrolysis of soybean protein with low-phytate content on the iron binding ability of the hydrolysate. In this study, SPI hydrolysates with low-phytate content were enzymatically prepared by trypsin. The effects of chemical modifications of SPI on the degree of hydrolysis and iron binding ability of hydrolysate, as well as the effect of molecular weight distribution on iron binding capacity of hydrolysate, were investigated.

MATERIALS AND METHODS

Materials: SPI was purchased in February 2011 from local Food Market, Hangzhou city, Zhejiang Province, China. It was immediately transported to the Lab of Food Science and Technology, China Jiliang University and stored at -20ºC until used. Trypsin (2, 500 U/mg), Amberlite 717, succinic acid, succinic anhydride and Sodium Trimetaphosphate (SMTP) were purchased from Shanghai Chemical Reagent Co. Ltd., China. Bovine serum albumin was purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals used in the experiments were from commercial resource and of analytical grade.

Phytate removal of SPI: Phytate was removed from the SPI using anion exchange resin (Amberlite 717) by the method of Kumagai *et al*. (2004) with some modifications. In simply, the anion exchange resin was washed successively with 1.0 N HCl, distilled water, 1.0 N NaOH, distilled water and 50 mM pH 7.4 Tris-HCl buffer, respectively. The soy protein isolate (0.5%, w/v) was suspended in 50 mM Tris-HCl buffer-resin solution and stirred at 4ºC for 2 h. Then the mixture was filtered through duplex cotton cloth and the filtrate was dialyzed overnight against distilled water at 4˚C. The contents were collected and lyophilized.

Chemical modification of SPI: The phytate-removal SPI solution (5%, w/v) was prepared and then was mixed with different content of Citric Acid (CA), Acetic Acid (AA), Hydrochloric Acid (HA), STMP, Sodium Sulfate (SS), Succinic Acid (SA) or Succinic Anhydride (SAH) to form suspensions. The suspensions were incubated for 2 h in a water bath shaker at room temperature. Then the suspension was centrifuged (10, 000 g, 10 min, 4ºC) and the supernatant was dialyzed against distilled water at 4ºC for overnight to remove the ammonium, phosphors or other ions and then freeze-dried. SPI without chemical modification under the same conditions was used as the control.

Preparation of SPI hydrolysate: Trypsin was chosen to hydrolysis SPI to produce bioactive peptides. The hydrolysis conditions were: temperature of 37ºC, time duration of 120 min, pH 8.0 and E/S of 1:50. A 50.0 g SPI powder was homogenized with 1.0 L of buffer solution (50 mM Tris-HCl, pH 8.0) and the homogenate was preheated 10 min at 37ºC then adding 1.0 g trypsin to the mixture to begin hydrolysis. At the end of hydrolysis, the solution was boiled for 10 min to inactivate the enzyme and then centrifuged at 8,000 g for 10 min at 4ºC. The supernatant was collected for further study.

Determination of protein content: The protein contents were determined by Folin-phenol method (Lowry *et al*., 1951) using Bovine Serum Albumin (BSA) as standard.

Degree of hydrolysis: The Degree of Hydrolysis (DH) was defined as the percentage of peptide bonds cleaved during a reaction and calculated from the ratio of free amino groups to the total number of peptide bonds in soluble factions, according to the method described by Jens (1979):

DH (%) = 100 ×[soluble protein content/total protein protein]

Determination of iron binding ability: Iron binding ability of SPI hydrolysate was determined by a modification of the ferrozine method (Carter, 1971). A 2 mL peptide (800 μg/mL) and 2 mL of distilled water were mixed with 100 μL 1 mM $FeSO_4$. After 10 min, 200 μL 5 mM ferrozine was added and mixed well. The soluble iron was determined.

Ultrafiltration of SPI hydrolysate: SPI hydrolysates were collected and subjected to ultrafiltration membranes system with molecular weight cutoff of 10, 5 and 3 kDa, respectively. Four peptide fractions with different molecular weight ranges were prepared:

- Fraction I (>10 kDa)
- Fraction II (5-10 kDa)
- Fraction III (3-5 kDa)
- Fraction IV (<3 kDa)

The peptide fractions were lyophilized and stored at -50ºC until use.

Amino acid composition analysis: The total amino acid composition was determined according to the method of Heu *et al*. (2003). The protein samples were placed in ampoules and mixed with 6 N HCl. After sealing the ampoule under vacuum, the samples were hydrolyzed at 110ºC for 24 h. The sample were diluted, filtered and loaded on a Modle S433D automatic amino acid analyzer (Sykam Corp., Eresing, Germany) for amino acid analysis.

Statistical analysis: The sample treatments in the present study were run in triplicate and all analyses were

Table 1: Effects of chemical modification on the degree of hydrolysis (DH) and iron binding ability

Methods of chemical modification	Protein content (mg/mL)	DH (%)	Iron binding ability (μg/mg-protein)
Control	6.53±0.07^{c}	18.07±0.65^{d}	18.47±0.13^{b}
CA	7.16±0.19^{a}	18.41±0.25^{cd}	14.90±0.18^{e}
AA	7.26±0.20^{a}	20.37±0.14^{a}	16.09±0.48^{d}
HA	7.15±0.14^{a}	18.10±0.36^{d}	14.15±0.50^{f}
STMP	6.06±0.10^{d}	17.86±0.07^{d}	20.16±0.36^{a}
SS	6.54±0.06^{c}	18.71±0.39^{c}	17.52±0.27^{c}
SA	5.92±0.22^{d}	19.42±0.20^{b}	1.15±0.59^{h}
SAH	6.82±0.13^{b}	15.87±0.39^{e}	6.79±0.58^{g}

The values are Mean D of triplicate measurement; Values with different letters in the same column are significantly different (p<0.05); CA: Citric acid; AA: Acetic acid; HA: Hydrochloric acid; STMP: Sodium trimetaphosphate; SS: Sodium sulfate; SA: Succinic acid; SAH: Succinic anhydride

performed in duplicate. Data were expressed as means±standard deviations (SD). The differences between the means of the treatments were compared by one-way ANOVA at a significance level of p<0.05. Statistical analyses were performed with the SPSS package (SPSS 13.0 for windows, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Chemical modification: Chemical modifications of soybean protein products to change their properties have become increasingly popular as a research area in the last twenty years. The purpose of these modifications include the reduction of phytate content, the reduction of protease activity, the increase or decrease in protein solubility to obtain better functional properties of solubility-dependent, the increase in nutritive value by covalent binding of amino acids with sulfur, and the elimination undesirable odor or flavor. The methods of chemical modifications include acid-alkali denaturation, chemical inactivation of protease inhibitors, reduction of phytate concentration, succinylation, acetylation, maleylation and phosphorylation, etc. The effects of acid treatments, succinylation, acetylation and phosphorylation on the iron binding ability of phytate-removal SPI trypsin hydrolysate were shown in Table 1. Acetic acid treatment can increase significantly the DH of SPI hydrolysate, but did not increase the iron-binding ability. Phosphorylation by STMP can increase significantly the iron-binding ability and did not change the degree of hydrolysis. Phosphorylation is one of the most important modifications of food proteins to increase in solubility and decrease in pI of the proteins, thereby changing the functional properties, especially nears the pI of the original proteins. Because phophate groups can be attached to the oxygen of seryl, threonyl, aspartyl (β-carboxyl) and tyrosyl residues and via nitrogen to lysyl (ϵ-amino) and histidyl (1 and 3) residues. STMP had been reported to be very effective phosphorylation reagent of SPI (Lee *et al*., 2005). The phosphorylation conditions with STMP, including temperature, pH, addition quantity and time, would affect the DH and iron binding ability of

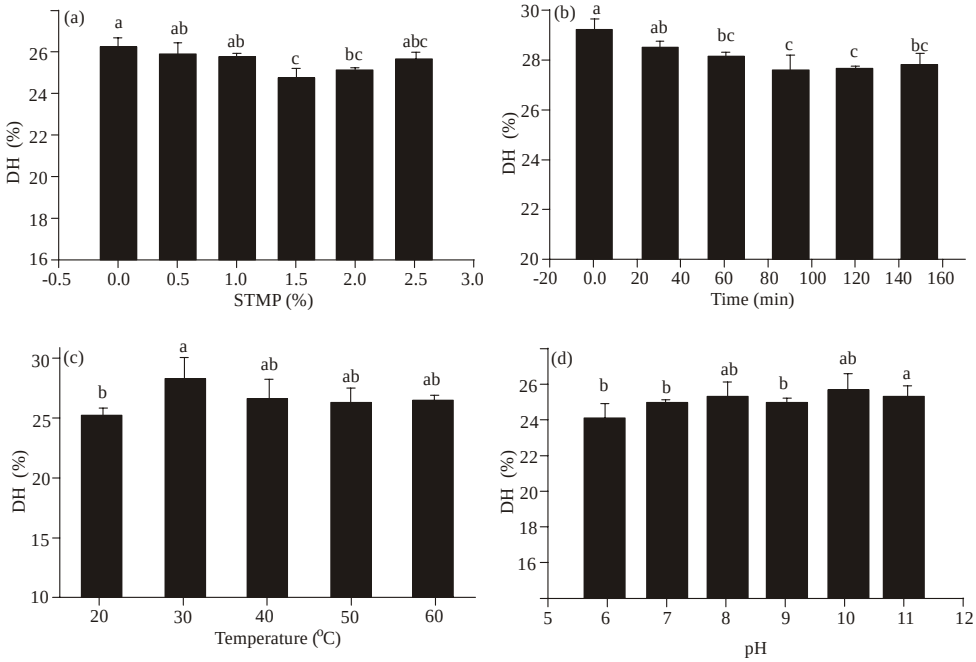

Fig. 1: a, b, c, d: Effects of phosporylation conditions with Sodium Trimetaphosphate (STMP), STMP concentration (a), time (b), temperature (c) and pH (d), on the degree of hydrolysis (DH) of Soybean Protein Isolate (SPI) hydrolysate. Values with different letters are significantly different (p<0.05)

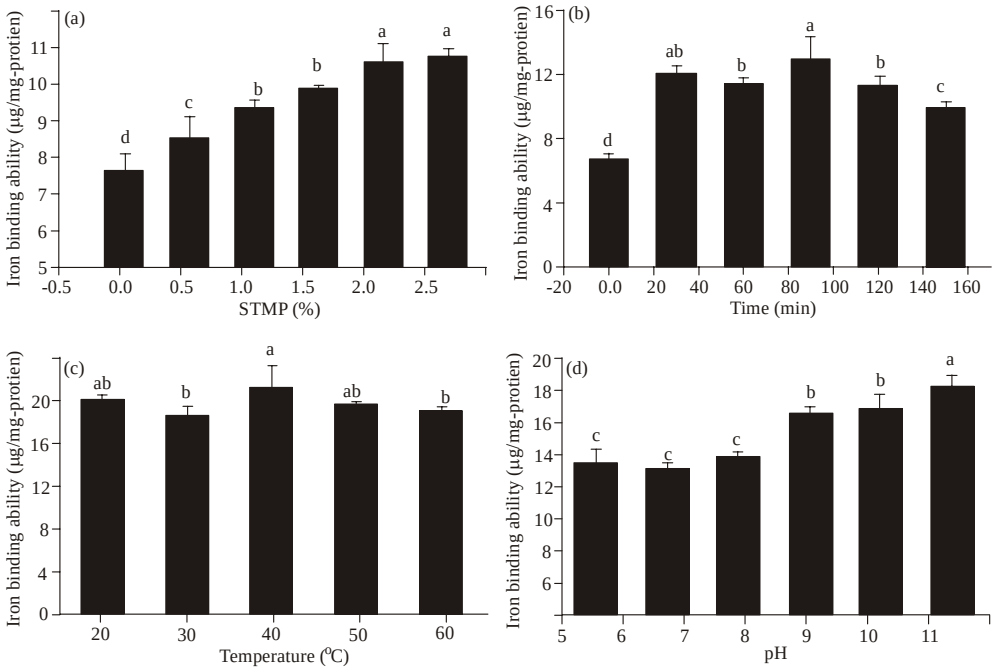

Fig. 2: a, b, c, d: Effects of phosporylation conditions with Sodium Trimetaphosphate (STMP), STMP concentration (a), time (b), temperature (c) and pH (d), on iron binding ability of Soybean Protein Isolate (SPI) hydrolysate. Values with different letters are significantly different ($p<0.05$)

the phytate-removal SPI hydrolysate. The reaction conditions were optimized, in order to satisfy iron binding ability of SPI hydrolysate, as shown in Fig. 1 and Fig. 2. The reaction conditions did not improve significantly the DH of hydrolysate (Fig. 1). The iron binding ability of phosphorylation SPI hydrolysate was performed with increasing STMP concentrations, at pHs of 6 to 11. As shown in Fig. 2a, the addition of STMP up to 2.5% (w/v) induced a proportional augmentation in the iron binding ability level, which might be caused by the increase in phosphorylation levels. Our result was in agreement with the data obtained in a recent report (Lee *et al*., 2005), which the calcium binding ability of SPI peptides was improved with STMP addition. The phosphorylation reaction time of 20 min was sufficient for improving the iron binding ability, as shown in Fig. 2b. Alkaline conditions (pH 9.0 or higher) were beneficial of phosphorylation reaction, which improved the iron binding capacity of phytate-removal SPI hydrolysate (Fig. 2d).

Molecular weight distribution and amino acid compositions: The relationship between the molecular weight distribution of protein hydrolysate and their mineral binding property or enhance of mineral absorption is not fully understood. It has been reported that a smaller fragment of less than 1kDa phosphopeptides derived from hen yolk phosvitin is unable to bind calcium to a significant extent (Jiang and

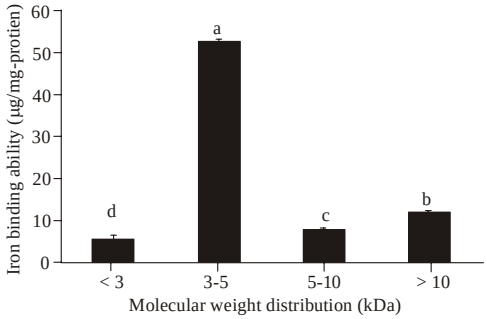

Fig. 3: Effects of molecular weight distribution on iron binding ability of Soybean Protein Isolate (SPI) hydrolysate. Values with different letters are significantly different ($p<0.05$)

Mine, 2001). Lee and Song (2009) purified an iron binding nona-peptide (1055 Da) from hydrolysates of porcine blood plasma protein. However, tripeptide and hexapeptide from beta-casein enhanced iron absorption significantly by Caco-2 cells (Chaud *et al*., 2007). In the present study, the effects of the molecular size of the phosphopeptides derived from phosphorylated SPI and its subsequent trypsin hydrolysate were studied. The hydrolysates were ultrafilterated into four fractions with molecular weight cutoff of 10, 5 and 3 kDa, respectively. The iron binding abilities of the four fractions were determined, as shown in Fig. 3. The fraction III with

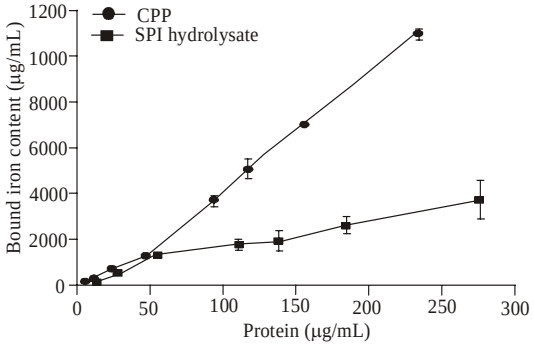

Fig. 4: The iron binding abilities of Soybean Protein Isolates (SPI) hydrolysate and Casein Phosporylation Peptide (CPP). Values with different letters are significantly different ($p<0.05$)

Table 2: Amino acid composition of soybean protein isolate (SPI) and peptide fractions by ultrafiltration of trypsin hydrolysate

Amino acid	SPI (%)	Fraction I (%)	Fraction II (%)	Fraction III (%)	Fraction IV (%)
Asp	11.3	11.1	11.2	10.5	8.3
Thr	4.1	3.3	3.8	3.7	4.1
Ser	6.4	5.1	5.8	6.6	7.6
Glu	22.5	21.9	16.1	14.5	9.0
Gly	6.9	7.0	7.2	6.9	6.9
Ala	6.0	5.7	7.2	7.4	8.3
Cys	2.3	1.5	0.8	1.0	4.1
Val	5.1	4.8	5.3	5.3	6.2
Met	0.9	1.0	1.0	1.0	0.7
Ile	2.3	4.7	5.1	5.6	5.5
Leu	8.0	7.9	9.4	9.8	9.7
Tyr	2.5	2.4	3.1	3.3	3.4
Phe	4.4	3.7	5.1	5.4	8.3
Lys	5.3	5.5	5.4	5.7	6.9
His	2.1	2.1	1.9	2.1	0.0
Arg	5.5	6.0	5.6	5.9	6.9
Pro	4.4	6.3	5.9	5.2	4.1
Total molar ratio	100.0	100.0	100.0	100.0	100.0

The molecular weight distribution of four fractions were Fraction I (>10 kDa), Fraction II (5-10 kDa), Fraction III (3-5 kDa) and Fraction IV (<3 kDa), respectively

molecular size of 3-5 kDa showed highest iron binding capacity.

Caseinophosphopeptides (CPP), which contain binding sites, phosphoseryl and carboxyl, for different minerals, have been widely studied among metal-binding peptides. In present study, the iron binding ability of the fraction III of phytate-removal SPI hydrolysate with molecular size of 3-5 kDa was compared with that of CPP at different concentrations, as shown in Fig. 4. At low peptides content, the SPI peptides had the same iron binding ability as CPP. However, at high peptides content, CPP had higher iron binding capacity that SPI peptides.

The amino acid compositions of SPI trypsin hydrolysate and four ultrafileration fractions were shown in Table 2. There were no significant different in amino acid compositions between the four fractions. But the fraction III with molecular size of 3-5 kDa exhibited higher iron binding capacity than that of other fractions (Fig. 3), which might be related to its higher phosphorylation level.

CONCLUSION

Chemical modifications affected the iron binding capacity of phytate-removal SPI trypsin hydrolysate. Phosphorylation with STMP increased significantly the iron binding ability of SPI hydrolysate. Several fractions with different molecular size exhibited obvious different iron binding ability. The middle molecular size (3-5 kDa) fraction had highest iron binding ability.

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