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In Vitro Antioxidant Activities of Protein Hydrolysate from Germinated Black Soybean (*Glycine max* L.)

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Abstract: During this study, the effect of germination in combination with in vitro pepsin and pancreatin digestion of protein flours extracted from black soybean on the production of bioactive peptides was investigated. Black soybean (*Glycine max* L.) were germinated for 3 days (at 24, 48 and 72 h, respectively germination periods) and harvested. Protein hydrolysates from germinated black soybean were prepared from protein isolate by in vitro digestion using pepsin and pancreatin and then evaluated for antioxidant activity. Soy protein hydrolysate from 48 h of germination exhibited the highest scavenging activity against 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) free radicals (76.56 % at 2.5 mg/mL) followed by 24 and 72 h (66.62 and 60.55% at 2.5 mg/mL, respectively). Protein hydrolysate from germinated black soybean also exhibited noticeable scavenging activity for hydroxyl. Soy protein hydrolysate from 48 h of germination (97.475% at 2 mg/mL) significantly was the most effective in neutralizing •OH ($p<0.05$) than that from 24 and 72 h (88.56 and 92.5%, respectively at 2 mg/mL), compared to the nongerminated which was 34.42% also at the same concentration. In addition, soyprotein hydrolysate from 24 h of germination significantly showed the highest reducing power (0.32 at 2.0 mg/mL) compared to that from 48 and 72 h germination period (0.25 and 0.23 at 2.0 mg/mL, respectively). Molecular Weights distribution of protein hydrolysates from germinated black soybean was believed to have correlation with their antioxidant activities. Results showed that germination with *in vitro* digestion (pepsin and pancreatin) of protein hydrolysate from germinated black soybean was successful in production of natural antioxidant compounds which established obvious antioxidant potency than non-germinated. As conclusion, soy protein hydrolysate from 48 h of germination had the best antioxidant potency and could be used as natural antioxidant in food systems.

Keywords: Antioxidant activity, black soybean, germination, *in vitro* digestion, molecular weight distribution, protein hydrolysate

INTRODUCTION

Nowadays, there is a wide interest in the effects of processing on the antioxidant compounds of legumes. Indeed, many bioactive compounds with antioxidant activity were present in legume seed. The bioactive compounds present in the soybean seed can be divided into non-protein compounds, such as isoflavones, saponins and protein compounds, such as Bowman-Birk inhibitors, lunasin and others. Biologically active peptides and proteins are either naturally produced by enzymatic digestion, fermentation, germination or enzymatic hydrolysis. Germination can cause significant changes in the biochemical characteristics of seeds and during this process, storage proteins can be degraded by proteases. It can increase its nutritional value, while it improves protein digestibility, reduces anti-nutritional factors and hydrolyses oligosaccharides (raffinose and stachyose) (Bau *et al*., 2000). It can also lead to modification of bioactive constituents (Paucar-Menacho *et al*., 2010).

Hydrolysis is a process that can release shorter chain compounds with lower molecular mass. Moreover, soy protein hydrolysates are physiologically better than intact proteins, because their intestinal absorption appears to be more effective due to the increased solubility of proteins (Kong *et al*., 2008) and also were considered as a potential dietary source of natural antioxidants with important biological activities (Darmawan *et al*., 2010). Hydrolysis of common bean, such as soybean, isolated protein result in the production of peptides with better antioxidant and anti-inflammatory properties (Oseguera-Toledo *et al*., 2011). The benefits of food antioxidants have been widely reported (Psaltopoulou *et al*., 2011) and the ability of peptides to behave as antioxidants is

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attributed to their capacity to interact with free radicals (Xiong, 2010). To realize hydrolysis, previous studies have demonstrated that pepsin and pancreatin are commonly used to produce soybean hydrolysates with biological activity (Darmawan *et al*., 2010). Black soybean (*Glycine max L*.), a variety of soybean with a black seed coat, that belongs to the family of Leguminosae, has been widely used as a tonic food and material in oriental medicine for hundreds of years. Numerous studies have reported higher levels of nutrients, such as amino acids, digestive protein, available carbohydrates and other compounds and lower levels of non-nutritive factors in legume sprouts as compared to non-germinated seeds (Vidal-Valverde *et al*., 2002).

In recent years, there is a growing interest to identify and utilize anti-oxidative compounds in many natural sources, such as soy protein (Moure *et al*., 2000). In spite of the physiological importance, the traditional Chinese medicine theory believes that black soybean has been used as a component in ancient medicines to treat diabetes, hypertension, anti-aging, cosmetology, blood circulation and so on Cho *et al*. (2001) because of its active peptide compounds. Adipogenesis inhibitory peptide was isolated and identified from black soybean protein hydrolysate (Kim *et al*., 2007a). Only a couple of studies, to the best of our knowledge, had been performed to evaluate antioxidant properties of protein hydrolysates prepared from germinated black soybean. From these studies, it had been found that the formations of bioactive compounds with more potent antioxidant activity were obtained by combination of soybean germination and Alcalase hydrolysis (Vermont *et al*., 2012).

The aim of this study was to determine the effect of germination in combination with *in vitro* pepsin and pancreatin digestion of protein flours extracted from black soybean on the production of bioactive peptides; therefore the antioxidant activity profile of the bioactive peptide was investigated. Furthermore, Molecular Weight (MW) distribution was also evaluated to determine their relationship with the antioxidant properties.

MATERIALS AND METHODS

This study was conducted in the state Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, PR China between Septembre 2011 and Mai 2012.

Materials: Black soybean seeds (*G. max L*.) with green cotyledons, were purchased from a local supermarket in Wuxi, Jiangsu Province, China. Enzymes (pepsin and pancreatin) and 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co., Shanghai, China. The other solvents/chemicals used were of analytical grade obtained from Shanghai Chemical Reagent Co., Ltd., Shanghai, China.

Preparation of sample: The seeds were germinated according to Se-Kwon *et al*. (2011) method for three days and were harvested everyday (24/48/72 h), ground into flour, which was dried then stored in a dessicator for further processing. The fine flour of germinated soybean was passed through a 60-mesh sieve and defatted by n-hexane extraction (soy flour/hexane = 1:5, v/v) for 10 h at room temperature. The oil-free flours were desolventized in open air then stored for further analysis.

Preparations of Black Soybean Protein Isolate (BSPI): The protein isolate was prepared according to the method described by Chang-Qing and Hai-We (2008). The defatted soybean flour was extracted for 3 h at room temperature with water and adjusted to pH 8.0 with 2 N NaOH [water: flour ratio, 10:1 (v/w)]. The mixture was centrifuged at 5000 g for 20 min at 4°C. After centrifugation, The supernatant was adjusted to pHi (pH isoelectric) 4.5 with 1 N HCl and then kept for 2 h at 4°C and subsequently centrifuged at 5000 g for 20 min at the same temperature. The precipitate was then washed twice with distilled water, neutralized to pH 7.0 with 2 N NaOH at room temperature and then freeze-dried. The dried protein was stored in dessicator at room temperature for subsequent analysis. The protein content of all samples was determined by Kjeldahl method (AOAC, 1995).

Preparation of Black Soybean Protein Hydrolysate (BSPH): Protein isolate were hydrolysed by sequential treatment with pepsin and pancreatin according to Lo *et al*. (2006). A sample was dissolved in distilled water to give a ratio of 1:5 (w/w). The first hydrolysis was with pepsin (1%) (pH 2.0) for 30 min and was stopped by increasing the pH to 7.0. Then, the pancreatin (2%) was added (pH 7.0) at the same temperature 37°C for another 30 min. The pH of the mixture was adjusted with 0.1 mol/L NaOH and 0.1 mol/L HCl during the hydrolysis. The hydrolysis reaction was stopped by heating in the thermostat-controlled water bath for 15 min at 95°C to inactivate proteases. The hydrolysate solution was centrifuged at 4000×g for 10 min after cooling down to room temperature. The supernatants were stored at -20°C low temperature for further analysis.

DPPH radical-scavenging activity: It is known that DPPH radical scavenging activity test system is simple to measure and can be used for the primary characterization of the scavenging potential of compounds (Nagai *et al*., 2002; Zhu *et al*., 2006). Therefore, the scavenging activity of the three samples hydrolysate on the DPPH free radical were measured according to the method described by Se-Kwon *et al*. (2011) and Suda (2000). The reaction mixture contained 2.0 mL of each sample, 2.0 mL of phosphate buffer (0.02 mol/L, pH 6.0) and 2.0 mL of 0.2 mmol/L DPPH in 95% ethanol. This mixture was shaken. After incubation for 30 min at room temperature in the dark,

absorbance of the mixture was read at 517 nm against a blank by spectrophotometer. Experiments were carried out in triplicate.

The percentage scavenging effect was calculated as:

$$\text{Scavenging rate} = [1 - (A_1 - A_2) / A_0] \times 100$$

where,

A_0 : The absorbance of the control (without sample)
A_1 : The absorbance in the presence of the sample
A_2 : The absorbance without DPPH

Hydroxyl (•OH) radical-scavenging activity: The •OH radical scavenging assay was carried out using the method described by Jin *et al*. (1996). Both 1, 10-phenanthroline (5 mmol/L) and $FeSO_4$ (0.75 mmol/L) were dissolved in phosphate buffer (1 M; pH 7.4) and mixed thoroughly and 1.0 mL of H_2O_2 (0.01%) was then added. The mixture was incubated at 37°C for 60 min and its absorbance of was read at 536 nm. The percentage scavenging effect was calculated as:

$$\text{Scavenging rate} = [(A_1 - A_0) / (A_2 - A_0] \times 100$$

where,

A_0 : The absorbance of the control (hydrolysates were replaced by distilled water)
A_1 : The absorbance in the presence of the hydrolysates
A_2 : The absorbance without H_2O_2

Measurement of reducing power: The reducing power of the hydrolysates was measured according to the method described by Chen *et al*. (2007). Different concentrations of protein fractions were mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 1.0% potassium ferricyanide. The mixtures were incubated at 50°C for 20 min. After incubation, the reaction was terminated by the addition of 2.5 mL of 10% Trichloroacetic Acid (TCA) and then followed by centrifugation at 5000×g for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) Ferric Chloride ($FeCl_3$) and then the absorbance of the mixture was measured at 700 nm against a blank. Increase of absorbance of the reaction mixture at a wavelength of 700 nm indicates an increase of reducing power.

Determination of molecular weight distribution: The protein hydrolysate from germinated soybean flour (24/48/72 h) was analyzed for Molecular Weight distribution using a Waters TM 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA). The hydrolysates were loaded onto TSK gel G2000 SWXL column (I/d/7.8×300 mm, Tosoh, Tokyo, Japan), eluted with 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 mL/min and monitored at 220 nm. A molecular weight calibration curve was obtained from the following standards from Sigma: cytochrome C (12,500 Da), aprotinin (6500 Da), bacitracin (1450 Da), tetrapeptide GGYR (451 Da) and tripeptide GGG (189 Da). Results were processed using Millennium 32 Version 3.05 software (Waters Corporation, Milford, MA 01757, USA). 2.12.

Data analysis: Results were expressed as the mean±standard error, Standard deviation was also calculated. Statistical analysis of the data was carried out by Duncan's multiple comparison test ($p<0.05$) Analyses were done using the Excel program (Microsoft) and the SPSS software package version 10.0 of SPSS Inc. (Chicago, IL, USA.)

RESULTS AND DISCUSSION

Protein content of germinated soybean flour and its protein isolate and hydrolysate: The protein content of the germinated soybean was summarized in Table 1. Ghavidel and Prakash (2007) have studied on the effect of germination of legumes and have found that germination can increase protein content. The result showed that after the first day of germination, the protein content significantly ($p<0.05$) increased by 16.85 and 15.41%, respectively for defatted flour and the protein extracted isolate. Second day of germination showed that the protein extracted from defatted flour, protein extracted isolate and protein hydrolysate were slightly reduced by 8.74, 4.82 and 9.89%, respectively. However, the decrease was not significant for the second to the third day (by 0.37, 1.38 and 2.15%, respectively). Those findings were similar to that of Bordignon *et al*. (1995) who noticed that the protein content studied reached the maximum values before 48 h germination. Moreover, Bau *et al*. (1997) showed that this increase might be possibly due to the synthesis of enzyme proteins or a compositional change following the degradation of other constituents. However, the loose of protein could be explained by protein synthesis that occurred during imbibitions, also hormonal changes played an important role in achieving the

Table 1: Protein content of germinated soybean flour and its protein isolate and hydrolysate

	Protein content (%) /germination time			
Sample	0 h	24 h	48 h	72 h
Defatted flour	37.19±0.75[a]	43.46±0.25[c]	39.66±0.50[b]	39.81±0.40
Protein isolate	69.55±0.55 [a]	80.27±0.67[c]	76.40±0.25 [b]	75.46±0.66
Protein hydrolysate	77.50±0.60[a]	97.28±1.40[c]	87.65±0.73[b]	85.76±0.77

Each value represents the mean of three independent experiments; Protein content (N×6.25); Means in a row with different letter were significantly different, according to Turkey test $p<0.05$

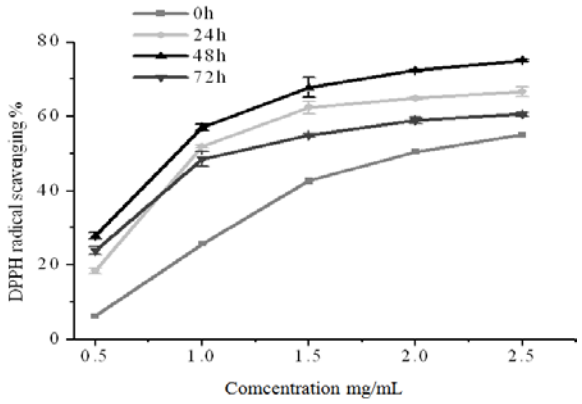

Fig. 1: DPPH radical scavenging activities of the different protein hydrolysates from the germinated soybean flour (24/48/72 h) compared to the non-germinated flour (0 h) at different concentrations
Each value is expressed as mean±S.D.

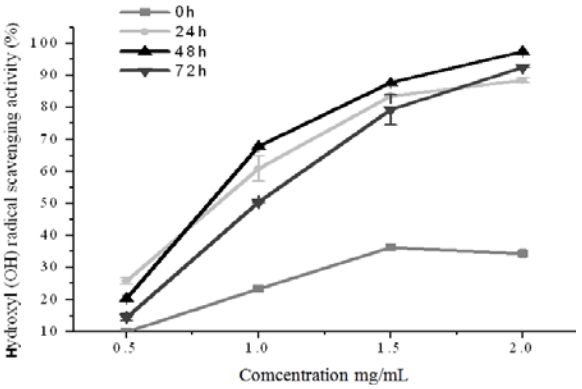

Fig. 2: Hydroxyl (OH) radical scavenging activities of the different protein hydrolysate from the germinated (24/48/72 h) soybean flour at different concentrations
Each value is expressed as mean±S.D.

completion of germination according to Nonogaki *et al*. (2010). The protein content of the germinated soybean after hydrolysis was also shown in Table 1. After hydrolysis the protein content was significantly increased by 21.19, 10.39 13.64% for 24, 48 and 72 h, respectively. This increase might be possibly due to the breaking bond during the hydrolysis. Furthermore, the drop in protein content seems to indicate that proteolysis outpaces protein synthesis in the growing sprouts (Rodriguez *et al*., 2008).

DPPH radical-scavenging activity: The DPPH radical is a stable organic free radical with an adsorption peak at 517 nm. Adsorption disappears when accepting an electron or a free radical species, which results in a noticeable discoloration from purple to yellow (Liu *et al*., 2009). As shown in Fig. 1, the DPPH radical scavenging activities of all the extracts were influenced by the concentration. The radical scavenging activities increased significantly ($p<0.05$) for all samples. The DPPH scavenging activity of the extracts followed the following order: 48>24>72>0 h. Among the different hydrolysates, 48 h exhibited the highest radical scavenging activity value (76.56% at 2.5 mg/mL) followed by 24 h (66.62% at 2.5 mg/mL) and 72 h (60.55% at 2.5 mg/mL), while the lowest DPPH radical-scavenging activity was obtained with non germinated 55.02% sample at the same concentration. When DPPH radicals encounter a proton-donating substance such as an antioxidant, the radicals would be scavenged and the absorbance is reduced (Jao and Ko, 2002). The differences in the radical scavenging ability found here might be attributed to the difference in amino acid composition and/or molecular weight distribution of peptides within protein hydrolysates.

Hydroxyl (OH) radical scavenging activity: It is well known that the radical system used for antioxidant evaluation may influence the experimental results and two or more radical systems are required to investigate the radical-scavenging capacities of a selected antioxidant (Yu *et al*., 2002). The hydroxyl radical-scavenging abilities of different protein hydrolysate from the germinated (24/48/72 h) soybean flour at different concentrations are shown in Fig. 2. All hydrolysates showed relatively good hydroxyl radical-scavenging activity (between 88.56 and 97.475% at 2 mg/mL) compared to the control non-germinated 34.42%. Protein hydrolysate for the germinated 48 h (97.475%) significantly showed the higher ability to scavenge OH radicals than 24 h (92.5%) and 72 h (88.56%), respectively. Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal-ions, such as copper or iron. When a hydroxyl radical reacts with aromatic compounds, it can add on across a double bond, resulting in hydroxycyclohexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical, or decompose to phenoxyl-type radicals by water elimination (Lee *et al*., 2004). The finding in this study revealed that the fractions compounds must have some aromatic amino acid that reacting and leading to the reaction.

Reducing power activity: The reducing power activity, which may serve as a significant reflection of antioxidant activity, was determined using a modified Fe (III) to Fe (II) reduction assay; the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of the samples. The presence of antioxidants in the samples causes the reduction of the Fe^{3+}/Ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring of the formation of Perls Prussian blue at 700 nm (Ferreira *et al*., 2007). Figure 3 presents the reducing power of the different protein hydrolysate from the germinated (24/48/72 h) and non-germinated (0 h) soybean flour. It was concentration dependent and

Table 2: Molecular weight distribution of protein hydrolysate from the germinated (24/48/72 h) and non-germinated (0 h)

Samples	>5 kDa	1-5 lDa	0.15-1 kDa	<0.15 kDa
0 h	27.98±0.77[a]	6.31±0.63[a]	10.60±0.41[a]	55.07±1.20[a]
24 h	19.18±0.88[c]	39.48±0.03[b]	23.99±0.50[b]	17.32±0.30[b]
48 h	22.97±0.04[b]	37.98±0.12	23.17±0.01	15.79±0.01
72 h	21.61±0.30	38.69±0.52	24.81±0.50[dsvc]	14.86±0.50

Means in a column with different letter were significantly different ($p<0.05$)

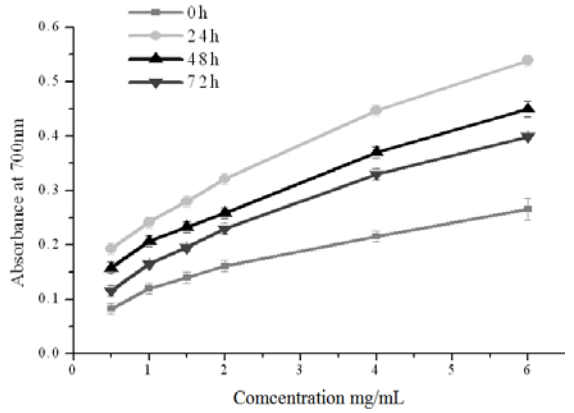

Fig. 3: Reducing power activity different protein hydrolysate from the germinated (24/48/72 h) and non-germinated (0 h) soybean flour at different concentrations
Each value is expressed as mean±S.D.

increased steadily with an increasing concentration of all samples, a similar result was found by Huda-Faujan *et al*. (2007). The reducing power of the samples followed the following order: 24>48>72>0 h the sample 24 h showed the highest reducing power. As well, at 2.0 mg/mL, the reducing power was 0.32 and while was found to be lower than wheat germ protein hydrolysates (0.51) prepared with Alcalase (Zhu *et al*., 2006), which was reported to possess excellent reducing power. It has been widely accepted that the higher the absorbance at 700 nm, the greater the reducing power. It had been indicated that a relation should be located between antioxidant activity and the reducing power (Yu *et al*., 2002). Similar observations had been reported with chickpea protein hydrolysate (Li *et al*., 2008a, 2008b). Samples with higher reducing power have better abilities to donate electron and free radicals to form stable substances, thereby interrupting the free radical chain reactions (Juntachote and Berghofer, 2005). The reducing power results in this assay revealed that protein hydrolysate from germinated soybean might have a good ability to donate electron, which was related to the antioxidant activity.

Molecular weight distribution: The molecular weight distribution of protein hydrolysate from the germinated (24/48/72 h) and non-germinated (0 h) soybean flour were analyzed (Table 2). The protein hydrolysate from non-germinated soy protein showed the highest percentage of large and low molecular weight fractions (>5 kDa, <0.5 kDa) 24.34 and 55.07%, respectively. The small proteins and native peptides found in legumes are prior to germination and they are subsequently used as a nutritional source for the young tissues (Zhang *et al*., 2006). While, during germination large molecular weight peptides were degraded into peptide with middle molecular weight (0.5-5 kDa). Consequently, the protein hydrolysate from germinated soybean with molecular weight 1-5 and 0.5-1 kDa significantly increased six times in percentages 39.48, 37.98, 38.69 and 23.99, 23.17, 24.81% for 24, 48 and 72 h, respectively. Molecular weight is an important parameter reflecting the hydrolysis of proteins, which further correlates with the bioactivity of protein hydrolysates (Li *et al*., 2008a). Pena-Ramos and Xiong (2001) reported that short peptides with molecular weight ranging from 370 to 1500 Da were responsible for the higher antioxidant activity whey protein hydrolysate in a liposome oxidizing system. Therefore, all the germinated samples showed higher percentage of peptides ranging between 370-1500 Da than non-germinated which assigned their higher antioxidant activity. Indeed these findings support the hypothesis that hypothesis that antioxidant activity is highly dependent on the molecular weight (Amadou *et al*., 2010).

CONCLUSION

Based on the findings obtained in this study, germination in combination with in vitro digestion (pepsin and pancreatin hydrolysis) of black soybean produced, more than three times in percentages, natural bioactive peptides (0.15-5 kDa). Therefore all protein hydrolysates from germinated black soybean flour are obviously better antioxidant than the one from non-germinated. The higher antioxidant activities observed has found to be related to the molecular weight distribution of peptide. Indeed, soy protein hydrolysate from germination 48 h showed the best antioxidant capacity but the nature of enzyme and the intestinal condition can always play important role in that issue. It could be used as natural antioxidants in food systems.

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