Antioxidant Properties of Peptides from Soybean Meal Protein Hydrolysates Evaluated by Electron Spin Resonance Spectrometry

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ABSTRACT
Peptides from soybean meal were subjected to extrusion and the effects of extrusion conditions, such as moisture of the materials, barrel temperature, screw speed and nozzle diameter, on the antioxidant properties were studied. The extrusion conditions that had the highest free radical-scavenging activity were further separated into three peptide fractions (using ultra filtration); hydrolysis of isolation of soybean meal protein (ISMP-I (> 3 kDa), ISMP-II (3 kDa - 1 kDa) and ISMP-III (< 1 kDa). The barrel temperature has the most influence on the antioxidant properties of the peptide, with a radical-scavenging activity (RSA) of 91.7%. Under the same extrusion conditions (moisture 10%; barrel temperature 105°C; screw speed, 100 r/min; and nozzle diameter, 18 mm), ISMP-III had the highest scavenging effects on hydroxyl radicals with an RSA of 99%. The antioxidant properties of the peptide from soybean meal protein hydrolysates could be enhanced by ultra-filtration.

KEYWORDS: Extrusion . Antioxidant . Soybean meal . Peptides

INTRODUCTION

Food with antioxidant helps us to resist the negative impact in daily lifestyle [1]. Antioxidants neutralize free radicals which help to oxidize our cells, as they have recently attracted considerable research interest, especially in the field of antioxidative peptides. Oxidative reactions in foods lead to the deterioration of quality attributes, such as flavor, aroma, texture and color. In particular, investigators report that free radicals, generated by oxidation, play a critical role in a variety of health disorders, including the processes of aging, diabetes mellitus, inflammation, and coronary heart and neurological disorders, such as Alzheimer’s disease [2, 3]. Soybean consumption is associated with reduced risk of cancer of the breast and prostate and may enhance survival [4, 5].

Soy protein is used as a functional ingredient in many different food products because it exhibits high nutritional and functional properties. Also, soy protein and soybean-derived peptides have many physiologic activities, particularly those related to the prevention of chronic diseases [6]. Peptides (ranging from 2 to less than 100 amino acid residues) with bioactive properties have been isolated from different protein hydrolysates obtained by enzymatic hydrolysis with protease. These peptides have exhibited several bioactive properties, including inhibition of bio-macromolecule peroxidation and elimination of free radicals produced in-vivo by enzymatic proteolysis of various food proteins [7]. Alfalfa leaf protein [8], silver carp [9], whey, casein, soy, and egg yolk hydrolysates have been shown to inhibit lipid oxidation in various muscle foods, such as beef and tuna [10, 11]. Cereal hydrolysates have been one of the most useful antioxidant products.
Extrusion is a rapid processing method involving a short period of high temperature and pressure and is used to prepare a variety of processed foods, such as baby foods, snack foods, ready-to-eat breakfast cereals and pet foods [12]. The effects of extrusion on the polyphenol content and antioxidant activity in rye bran chickpeas, corn, oats, carrots and hazelnuts [13, 14]. The effects of extrusion on the phenolic composition and antioxidant activity of kidney beans have been studied [15]. Although extrusion is a short cooking process, the temperatures encountered by the raw material in the barrel of the extruder are enough to induce changes in the major and non-nutritive components. The most significant changes are occurring in cereal starch and protein that contribute to the structure, texture, mouth feel and bulk density [12].

The technique of electron spin resonance (ESR) is a sensitive method for assessing the free radical scavenging activity of antioxidants [16]. This method is able to provide information about the conformational properties of peptides and their interaction with macromolecules and the membrane of biological interest using stable free radical spin labels.

The objective of this study was to investigate the extrusion effects on the antioxidant properties of a peptide from soybean meal protein hydrolysates evaluated by electron spin resonance spectrometry. The most potent (by ant oxidative activity) protease hydrolysates were fractionated using ultra filtration membranes. The antioxidant properties of the peptide fractions on hydroxyl radicals were investigated using ESR spectroscopy.

MATERIALS AND METHODS

Materials:
Soybean meal and flakes were purchased from local legumes store, Taif City, Kingdom of Saudi. The soybean flakes were cleaned, crushed and pressed from soybeans. Soybean oil was extracted using a solvent and desolventizing in a vacuum at 90°C. After the oil was extracted, the solvent was removed, and the flakes were dried, creating defatted soy flakes (soybean meal). The contents of the soybean meal and soybean flakes were tested on the basis of dry matter weight (Table 1).

Extrusion:
For extrusion, the motorized twin-screw extruder (45 kW of puissance and 380 V of maximal tension) has been used. The length of the screw was 1.47 mm, and the ratio of the length/diameter was 30 D. Raw granular materials were fed into the extruder at a mass rate of 45.4 kg h⁻¹. The temperatures of the six barrel zones were maintained at 45, 60, 75, 90 and 105°C, from the feeding port to the die section, throughout all experiments. The moisture content of the materials was 5-25% in feed. The screw speeds of the extruder were 60, 80, 100, 120 and 140 r/min. Five nozzles of different diameters (10, 15, 18, 22, and 28 mm) were used to press the materials. To monitor the temperatures and pressure of extruded products during processing, six thermocouple sensors were inserted along the barrel and in the die plate. A pressure transducer was also installed in the die plate. The feed rate, barrel temperature, die pressure and screw speed shown on the control panel were recorded. The extruded samples were collected for 5 min while the operating conditions were steady.

Preparation of peptide fractions:
Isolation of soybean meal protein:
Soybean protein isolate (SPI) was prepared from soybean meal at room temperature to prevent heat denaturation of the proteins [17]. The soybean meal was suspended in 100 mL of H₂O, at a pH of 8.5 and in a ratio of 1:10 (w/v); it was then stirred at room temperature for 1 h. Fiber was separated by centrifugation (5000 r/min 5°C). The supernatant was adjusted to pH of 4.5 with 2 M HCl to induce precipitation of soy proteins. The dispersion was centrifuged after 2 h at 4°C. The precipitate was washed with 10 mM sodium acetate buffer at a pH of 4.5 (1:8 ratio (w/v)) and centrifuged as described above; the supernatant from this washing step was discarded. The final precipitate (SPI) was adjusted to a pH of 7.0 and freeze-dried.

Production of protein hydrolysates from isolated proteins:
Alcalase protease was used for the hydrolysis of isolated soybean meal protein (ISMP). The ISMP was mixed with distilled water (5%, w/v) and homogenized at a speed of 10,000 rpm for 1 min. The homogenate was pre-incubated at 45°C for 20 min prior to enzymatic hydrolysis. The ISMP was hydrolyzed under enzymatic conditions (4.5 h; pH, 8.5 at 45°C). After hydrolysis, the sample was heated at 98°C for 10 min to inactivate the protease. The hydrolysate was centrifuged at 5000 g at 4°C for 15 min to separate the insoluble and soluble fractions. Finally, the supernatant was collected and lyophilized.

Separation of peptide fractions by ultra filtration:
Alcalase-hydrolyzed isolated soybean meal protein was fractionated into three different fractions using ultra filtration (8200, Amicon Corp., USA). The neutralized and filtered fraction was sequentially passed through Millipore membranes YM3 and YM1 (molecular mass cutoffs of 3 and 1 kDa, respectively) under 40
psi of nitrogen at 4°C, and aliquots were collected at each filtration step. Fractionates were designed as follows: ISMP-I (did not permeate the 3-kDa membrane), ISMP-II (permeated the 3-kDa membrane but not the 1-kDa membrane), and ISMP-III (permeated the 1-kDa membrane). All of the recovered fractions were lyophilized in a freeze drier.

**ESR spectroscopy:**
ESR spectroscopy was performed on a Brucker 300E. The conditions were as follows: center field strength of 351.194 mT, scan width of 10.00 mT, microwave frequency of 9.858 GHz, and power of 2.25 mW. DHX-IIIB ozone generator (China), a WTW dissolved oxygen meter (Germany) and a 10W GPH212T5 L/4 UV lamp (Germany) were used in the experiment.

**Hydroxyl radical-scavenging assay:**
Hydroxyl radicals were generated using the Fenton reaction system [18]. A peptide solution (50 μl) or the same volume of phosphate buffer (PBS) Ph 7.4 (0.2M Na₂HPO₄·0.3M NaH₂PO₄) was added to 50 μl of 0.3M DMPO (5,5-dimethyl-pyrrole N-oxide) and 50 μl of 10mM FeSO₄. The reaction was initiated by adding 50μl of 10mM H₂O₂. The reaction mixture was transferred to a quartz capillary tube, and the spectrum of the DMPO-OH adduct was recorded after 2 min using ESR spectrometry, as described above. The measuring conditions were as follows: magnetic field, 336.5±5 mT; power, 10 mW; modulation frequency, 100 kHz; amplitude, 1x1000; and sweep time, 2 min.

**Statistical Analysis:**
All extractions and determinations were conducted in triplicate, and the results were expressed on the basis of dry matter weight. The data are expressed as the means ±SD. The means were compared using the one-way and multivariate analysis of variance (ANOVA) followed by Duncan’s multiple range tests using SPSS 17.0. Significant differences between the individual means were accepted at $p < 0.05$.

**RESULTS AND DISCUSSION**

**Effect of barrel temperature on the hydroxyl radical-scavenging assay:**
Thermal processing is also known to alter the antioxidant profile and generate more antioxidants that can contribute to antioxidant activity. Increases in antioxidant activity due to thermal processing have been widely reported [19]. Antioxidant activity varied significantly when the extrusion was carried out at 105°C. The RSA ranged from 41.74% to 91.7% with the highest and lowest values occurring at 60°C. The highest antioxidant activity in an extruded bean/corn mixture at a temperature of 142°C and feed moisture of 16.5% [20]. Similarly, the extrusion process significantly increased the DPPH radical scavenging activity [21, 22].

**Effect of moisture content of materials on the hydroxyl radical-scavenging assay:**

When the extrusion temperature was kept constant (105°C) and the moisture content was increased from 5% to 25%, the RSA decreased significantly ($p < 0.05$) at 10, 15 and 20% moisture content of materials by 91.7%, 71.11% and 52%, respectively (Table 3). A change in the extrusion temperature and moisture could have resulted in the formation of different amounts of Maillard browning product [23]. Maillard browning is influenced by many factors, including temperature, reactant concentration, reaction time and water activity [24].

**Effect of the screw speed on the hydroxyl radical-scavenging assay:**
As presented in Table 4, the most influential factor on extrusion was the barrel temperature, but when the screw speed was 80r/min, the RSA was similar to the highest RSA of 90.16%. When maintaining the moisture content at 10% and the barrel temperature at 105°C while increasing the screw speed from 60 to 100r/min, the RSA showed a significant increase from 61.08% to 91.7%. With increasing screw speed, the RSA showed an opposite change, decreasing from 91.70% to 7.34%.

**Effect of nozzle diameter on the hydroxyl radical-scavenging assay:**
Utilizing optimal extrusion conditions for the barrel temperature at 105°C, moisture of the materials at 10% and screw speed at 100r/min while increasing the nozzle diameter from 10 to 28 mm, the RSA showed a significant increase that ranged from 20.57% to 91.7% (Table 5). The highest and the lowest increase were exhibited at nozzle diameters of 10 mm and 18 mm, respectively.

**Effect of different peptide fractions on the hydroxyl radical-scavenging assay:**
All peptide fractions exerted significant ($P < 0.05$) free radical-scavenging activity (Table 6). The RSA were 67.56%, 93.13% and 99.00% for ISMP-I, ISMP-II and ISMP-III, respectively. Figure 2 describes the ESR signal intensity of hydroxyl radical-scavenging assay. Among the three fractions, ISMP-II exhibited the highest...
inhibitory activity (P < 0.05); its antioxidant activity of RSA was nearly 100%. One explanation is that higher molecular weight peptides positively correspond to greater antioxidant activity. A study of five different peptide fractions mentioned that the smallest molecular weight peptide fraction exhibited the highest inhibitory activity, and its antioxidant activity was closer to that of the positive control, vitamin E, a well-known lipid-soluble natural antioxidant.

Table 1: Chemical Compositions of the Test Materials

<table>
<thead>
<tr>
<th></th>
<th>Protein</th>
<th>Fat</th>
<th>Soluble fiber</th>
<th>Insoluble fiber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean flake</td>
<td>36.73±0.17b</td>
<td>20.16±0.02b</td>
<td>1.20±0.01a</td>
<td>2.87±0.09b</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>47.46±0.07b</td>
<td>1.00±0.01b</td>
<td>1.03±0.15b</td>
<td>3.65±0.05b</td>
</tr>
</tbody>
</table>

Each value presented as the mean ± standard deviation (n=3). Data with different uppercase superscript letters in the same column of means respectively indicate significant difference (P < 0.05) analyzed by Duncan’s multiple range.

Table 2: The effect of barrel temperature on the hydroxyl radical-scavenging assay

<table>
<thead>
<tr>
<th>Barrel temp. (°C)</th>
<th>Moisture (%)</th>
<th>Screw speed (r/min)</th>
<th>Nozzle diameter (mm)</th>
<th>RSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>10</td>
<td>100</td>
<td>18</td>
<td>72.03b</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
<td>100</td>
<td>18</td>
<td>41.74a</td>
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<td>90</td>
<td>10</td>
<td>100</td>
<td>18</td>
<td>67.60a</td>
</tr>
<tr>
<td>105</td>
<td>10</td>
<td>100</td>
<td>18</td>
<td>91.77a</td>
</tr>
</tbody>
</table>

Each value presented as the mean ± standard deviation (n=3). Data with different uppercase superscript letters in the same column of means respectively indicate significant difference (P < 0.05) analyzed by Duncan’s multiple range.

Table 3: Effect of moisture content on the hydroxyl radical-scavenging assay

<table>
<thead>
<tr>
<th>Moisture (%)</th>
<th>Barrel temp. (°C)</th>
<th>Screw speed (r/min)</th>
<th>Nozzle diameter (mm)</th>
<th>RSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>105</td>
<td>100</td>
<td>18</td>
<td>64.07a</td>
</tr>
<tr>
<td>10</td>
<td>105</td>
<td>100</td>
<td>18</td>
<td>91.70a</td>
</tr>
<tr>
<td>15</td>
<td>105</td>
<td>100</td>
<td>18</td>
<td>71.11b</td>
</tr>
<tr>
<td>20</td>
<td>105</td>
<td>100</td>
<td>18</td>
<td>52.00b</td>
</tr>
<tr>
<td>25</td>
<td>105</td>
<td>100</td>
<td>18</td>
<td>54.29b</td>
</tr>
</tbody>
</table>

Each value presented as the mean ± standard deviation (n=3). Data with different uppercase superscript letters in the same column of means respectively indicate significant difference (P < 0.05) analyzed by Duncan’s multiple range.

Table 4: Effect of the screw speed on the hydroxyl radical-scavenging assay

<table>
<thead>
<tr>
<th>Screw speed (r/min)</th>
<th>Barrel temp. (°C)</th>
<th>Moisture (%)</th>
<th>Nozzle diameter (mm)</th>
<th>RSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>105</td>
<td>10</td>
<td>18</td>
<td>61.08c</td>
</tr>
<tr>
<td>80</td>
<td>105</td>
<td>10</td>
<td>18</td>
<td>90.16c</td>
</tr>
<tr>
<td>100</td>
<td>105</td>
<td>10</td>
<td>18</td>
<td>91.70c</td>
</tr>
<tr>
<td>120</td>
<td>105</td>
<td>10</td>
<td>18</td>
<td>46.18c</td>
</tr>
<tr>
<td>140</td>
<td>105</td>
<td>10</td>
<td>18</td>
<td>7.34c</td>
</tr>
</tbody>
</table>

Each value presented as the mean ± standard deviation (n=3). Data with different uppercase superscript letters in the same column of means respectively indicate significant difference (P < 0.05) analyzed by Duncan’s multiple range.

Table 5: Effect of nozzle diameter on the hydroxyl radical-scavenging assay

<table>
<thead>
<tr>
<th>Nozzle diameter (mm)</th>
<th>Barrel temp. (°C)</th>
<th>Screw speed (r/min)</th>
<th>Moisture (%)</th>
<th>RSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>105</td>
<td>100</td>
<td>10</td>
<td>20.57c</td>
</tr>
<tr>
<td>15</td>
<td>105</td>
<td>100</td>
<td>10</td>
<td>71.62c</td>
</tr>
<tr>
<td>18</td>
<td>105</td>
<td>100</td>
<td>10</td>
<td>91.70c</td>
</tr>
<tr>
<td>22</td>
<td>105</td>
<td>100</td>
<td>10</td>
<td>33.00c</td>
</tr>
<tr>
<td>28</td>
<td>105</td>
<td>100</td>
<td>10</td>
<td>79.69c</td>
</tr>
</tbody>
</table>

Each value presented as the mean ± standard deviation (n=3). Data with different uppercase superscript letters in the same column of means respectively indicate significant difference (P < 0.05) analyzed by Duncan’s multiple range.

Table 6: Results of different peptide fractions on the hydroxyl radical-scavenging assay

<table>
<thead>
<tr>
<th>Peptide fraction</th>
<th>RSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISMP-I</td>
<td>67.56a</td>
</tr>
<tr>
<td>ISMP-II</td>
<td>93.13b</td>
</tr>
<tr>
<td>ISMP-III</td>
<td>99.00c</td>
</tr>
</tbody>
</table>

Each value presented as the mean ± standard deviation (n=3). Data with different uppercase superscript letters in the same column of means respectively indicate significant difference (P < 0.05) analyzed by Duncan’s multiple range.
Fig. (1. a): ESR signal intensity of hydroxyl radical-scavenging assay for ISMP-I.

Fig. (1. b): ESR signal intensity of hydroxyl radical-scavenging assay for ISMP-II.

Fig. (1. c): ESR signal intensity of hydroxyl radical-scavenging assay for ISMP-III.
Conclustion:
Hydrolysates derived from soybean meal may serve as a good source of antioxidant peptides for nutraceutical and pharmaceutical ingredients. Extrusion conditions such as the moisture of materials, barrel temperature, and screw speed and nozzle diameter have an effect on the antioxidant properties of peptides from soybean meal protein hydrolysates. Though there was no specific rule indicating how each factor of extrusion affected the antioxidant properties of the peptide, we did determine for the first time that the barrel temperature is the most important factor, of the extrusion conditions examined in this study. When the moisture of materials was 10%, the barrel temperature was 105°C, the screw speed was 100 r/min and the nozzle diameter was 18 mm, the RSA showed the highest value of 91.7%. The extrusion conditions that showed the highest free radical-scavenging activity were further utilized to examine three peptide fractions for hydrolysis of isolated soybean meal protein (ISMP-I (>3kDa), ISMP-II (3kDa-1kDa) and ISMP-III (<1kDa). After ultra filtration, ISMP-III had the highest scavenging effects on hydroxyl radicals with a RSA of 99%.

Conflict of Interests:
The authors declare that there is no conflict of interest is regarding the publication of this paper.

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REFERENCES


