Comparative study on antioxidative activity of the seeds of hoary basil (Ocimum basilicum) protein hydrolysates produced by papain, pepsin and Protease G6 (alcalase)

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ABSTRACT

Excessive free radical production have known to be the cause of various degenerative disorder of human such as cardiovascular diseases, aging, cancer, and neurodegenerative disease like Alzheimer’s disease. By-products form the chemical extraction of essential oil and fiber of the seeds of hoary basil (Ocimum basilicum) are abundant and underutilized resources that can be used as a unique protein source to make protein hydrolysates. Antioxidative activity of protein hydrolysates prepared from hoary basil seed protein hydrolysates (HBPs) using papain, pepsin and Protease G6 (alcalase) were determined. In addition, all HBPs exhibited antioxidative activity in a concentration-dependent manner. Protease G6-HBPs generally showed greater antioxidative activity (0.0196 ± 0.064 mg/ml) than papain-HBPs (0.2033 ± 0.219 mg/ml), and pepsin-HBPs (0.0813 ± 0.00813 mg/ml) protein hydrolysates as indicated by the higher 1,1-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity. Protease G6-HBPs were fractionated into three fractions, HBPs-I (MW> 10 kDa), HBPs-II (MW = 5-10 kDa), and HBPs-III (MW < 5 kDa) and the in vitro antioxidant activities of all fractions were determined. HBPs-III showed the lowest IC₅₀ value at 0.02523 ± 0.14 mg/mL. In addition, the protein of hoary basil seed have a high percentage of essential amino acids, Moreover, protein showed the presence of phenylalanin, tyrosine, and histidine, these amino acids have also been reported to show antioxidant activity. HBPs had a high nutritional value and could be used as supplement to poorly balanced dietary proteins.

Keywords: Antioxidative activity, Protein hydrolysates, Hoary basil, Ocimum basilicum
1. INTRODUCTION

Oxidation is one of the main causes for diseases and pathogenesis in human. For example, free radical attack on proteins, lipids, and nucleic acids, results in cell damage, apoptosis and plays an important role in atherosclerosis, Alzheimer’s disease, inflammatory bowel disease, and certain cancers [1]. Essentially, all the cellular components and the specific constituents are susceptible to reactive oxygen species (ROS) and reactive nitrogen species (RNS), for example, hydroxyl radicals (OH), peroxyl radicals (OOR), superoxide anion (O$_2^-$), and peroxyl nitrite (ONOO$^-$). The accumulation of protein carbonyl compounds, a result of oxidation, is believed to be a main mechanism of aging process in human [2]. Antioxidants play a vital role in human body, reducing oxidative processes. In the human body, endogenous antioxidants, including enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and various nonenzymatic compounds such as selenium, α-tocopherol, and vitamin C help to protect tissues and organs from oxidative damage caused by ROS and RNS [3]. Apart of these, amino acids, peptides and proteins also contribute to the overall anti-oxidative capacity of cells and towards maintaining the health of biological tissues. In recent years, a considerable amount of research has also focused on the liberation of antioxidant peptides encrypted within food proteins, with a view to utilizing such peptides as functional food in gradients aimed at health maintenance. Interestingly, with the parent protein sequence, the peptides are inactive and thus must be released to exert an effect. These bioactive peptides are 2-20 amino acid residues in length, although some have been reported to have more than amino acid residues [4].

Protein hydrolysate is a product of hydrolysis reaction from protein. Mixtures of free amino acid are prepared by splitting a protein with chemical or enzyme. Chemical protein degradation is the low cost, but limited in its use in food product or pharmaceutical, make it difficult to control product quality [5]. The enzymatic hydrolysis by controlling condition such as hydrolysis time, temperature, pH, unit enzyme is a method that is more effective to provide the maximum among of peptides. Where with the enzyme has a specific substrate [6]. Enzymatic hydrolysis, which is also in a mild condition, can control degradation by choosing type of enzymes and optimized condition. The obtained protein hydrolysate was with desired properties. The source of protein hydrolysate is mostly waste or cheap materials from agricultural derived from plants or animals [7]. Protein hydrolysate from plants is the product of the hydrolyzed material that is high in protein as amino acids, peptides and other compound which can have aromatic ring types, such as phenylalanine, tyrosine, tryptophan, histidine and cysteine [8]. These amino acids can donate proton to the free radicals resulting this hydrolysate has an antioxidant activity. Reported low molecular polypeptide components with histidine, tryptophan and tyrosine showed highly antioxidant activity [9].

Hoary basil (Ocimum basilicum) is a biennial plant, one of the major oil producing species, commonly used in many kind of food. The seed are eaten as a dessert and used as a mild laxative. It can also decrease blood sugar levels in diabetes’s patients [10]. The initial study found that essential oil of Hoary basil composed of highly essential fatty acid [11]. Hoary basil seed is a by-product form the chemical extraction of essential oil and fiber. It is interested material in producing active protein hydrolysate. It increase value of waste and also, also it is reported that defatted Hoary basil seed was analyzed composed of 17.16% protein [11]. Although protein content of this seed less than the other, however we have trialed for the amount of active free amino acid before, other than hoary basil seed is a new source of protein hydrolysate. The hoary basil seed protein hydrolysates have not been study for bioactivity elsewhere. The goal of this study was to evaluate of antioxidant activity of bioactive protein hydrolysate by Protease G6 form hoary basil seed.

2. MATERIALS AND METHODS

Biological and chemical materials

Hoary basil seed were purchased from Youwarat market Bangkok Thailand. The samples were quickly taken to laboratory and kept in dark 4°C room until used. Ascorbic acid, bovine serum albumin (BSA), 2, 2-Diphenyl-1-pieryhydrazyl (DPPH), and trichloroaceticacid (TCA) were purchased from Sigma Chemicals Co. (USA). Protease G6 was purchased from Siam Victory Chemicals Co., Ltd (Thailand). All other unlabeled chemicals and reagents were of analytical grade.

Defatted hoary basil seed preparation

Raw hoary basil seed was selected and cleaned to remove contaminated, crushed with a blender and it was defatted by petroleum ether using soxhlet for 10-12 hour at 65°C. Hot air oven with 60°C then the defatted hoary basil seed was sieved using laboratory hammer mill through 90 meshes and was kept in the dedicator until used.
Total amino acid analysis

Acid hydrolysis

Five milliliters of HCl 6N was added (5 mg protein/ml HCl) and mixed. The tube was flushed with nitrogen for 1 min to remove air. Hydrolysis was then carried out at 110°C for 22 h. The internal standard (10 ml of 2.5 mM L-α-amino-n-butyric acid in HCl 0.1 M) was added and diluted with water to 250 ml. The solution was filtered with 0.20 µm filter and was then derivatized with 6 aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Flour reagent). It was then heated in a heating block at 55°C, for 10 min. Heating converts a minor side product of tyrosine to a major mono-derivatized compound. Total amino acid content was determined by high performance liquid chromatography.

Chromatographic conditions

Chromatographic separation was carried out in a Waters Alliance 2695 with heater amino acid analysis Hypersil Gold column C18. The column was thermostat at 35 ± 1°C and the flow rate was 1.0 ml/min. The injection volume was 5 µl. Mobile phase A consisted of sodium acetate buffer pH 4.90 and 60% acetonitrile.

Preparation of hoary basil seed protein hydrolysate (HBPs)

The sieved hoary basil seed less than 90 meshes was hydrolyzed using papain, pepsin, and ProteaseG6, and using a hydrolyze ratio of substrate: enzyme was 0.5:10 (w/v). The hydrolysate was conducted for 4 hours at 50°C with shaking 150 rpm. The reaction was stopped by heating at 90°C for 10 min. After that, centrifugation was used to keep supernatant at 15,000 × g, 15 min, 4°C. The protein content was determined by Bradford’s procedure.

Determine of degree of hydrolysis (DH)

The degree of hydrolysis was estimated by determination of free amino acid groups. Nitrogen content was analyzed by Kjeldahl method [12]. Samples were treated with 10% trichloroaceticacid (TCA) and centrifuge 15,000 x g for 30 min at 4°C. The supernatant was considered and %DH was calculated by formula below:

\[
DH\% = \frac{\text{Soluble Nitrogen in TCA} \times 100}{\text{Total Nitrogen in the sample}}
\]

Ultrafiltration

The protein hydrolysates were fractionated through ultrafiltration membranes using a bioreactor system (Amersham Biosciences, Sweden). From research showed that peptide which is a small molecules had antioxidant properties better than bigger, more over short peptides can be transform to use in pharmaceutical and industrial. HBPs peptide solution was pumped through a range of nominal molecular weight cutoff (MWCO) membranes of 10, and 5 kDa, respectively, in the order of decreasing pore size. MW > 10 kDa; retentate from 10 kDa membrane, MW = 5-10 kDa; retentate from 5 kDa membrane, and MW < 5 kDa; permeate from 5 kDa membrane were collected.

DPPH radical scavenging activity

The DPPH radical scavenging activity was modulated the previous method [13], sample with various concentration were mixed with 0.1 M DPPH in ethanol for 30 min prior measured by using spectrophotometer at 517 nm. The IC50 value (the concentration that causes a decrease in initial DPPH concentration 50%) was determined from the linear regression of the DPPH inhibition against the concentration of protein. The negative control (blank) was water instead of the protein / protein hydrolysate sample while the positive control was ascorbic acid (0 - 50 µg/mL). The percentage of radical scavenging was calculated as follows:

\[
\% \text{radical scavenging} = \frac{(Ac - Acb) - (As - Asb)}{(Ac - Acb)} \times 100
\]

where \( Ac \) is the absorbance of water plus DPPH (in methanol), \( Acb \) is the absorbance of the blank (water plus methanol without DPPH), \( As \) is the absorbance of the sample plus DPPH (in methanol) and \( Asb \) is the absorbance of the sample plus methanol without DPPH. Dilution of sample was used in order to obtain calibration curves and to calculate the IC50 values (IC50: concentration required to obtain a 50% radical scavenging activity).

Protein concentration

The protein content was determined [14]. Bovine serum albumin (BSA) was used as the standard with four different concentrations between 5-20 µg/ml to construct the calibration curve.

Statistical analysis

All investigations were done in triplicate. The results were indicated as the mean values ± stand deviation. GraphPad Prism (Version 6.00, GraphPad Software Inc, La Jolla, CA, USA) for windows was used to calculated
IC$_{50}$ values. Statistical tests of significant difference were performed by ANOVA, with $p < 0.05$ being accepted as significant.

### 3. RESULTS

**Amino acid content of hoary basil seed**

Intact proteins, protein hydrolysates, individual peptides and amino acids have been reported to have significant antioxidant properties. Usually protein hydrolysates or peptide fractions showed greater antioxidant activity than intact proteins and amino acids. The antioxidant activities of protein digests vary depending on their peptide structure, ie., size of the peptides and their amino acid sequences, which are influenced by the source of protein and conditions of hydrolysis involved [15]. The relationship between hydrophobic amino acid and the reducing power of hydrolysates have been previously reported [16]. The reducing power assay is used to evaluate the ability of compound to donate electron or hydrogen to free radical thereby converting the radical to stable substances [17]. Data concerning qualitative and quantitative amino acids composition is presented in Table 1. Amino acid composition indicates the nutritional quality of protein. Glutamic acid and aspartic acid were found to be the major non-essential amino acids in the samples tested. Results indicated that all essential amino acids, except S-containing types and tryptophan, are present in high amounts in this specie. Moreover, protein showed the presence of phenylalanin, tyrosine, and histidine, these amino acids have also been reported to show antioxidant activity. Therefore, protein hydrolysates have antioxidant activity as well as high nutritive value because of the presence of important amino acids [15]. Results are comparable to those of earlier worker [18] while there is no previous report on amino acid composition of hoary basil seeds.

Table 1. Total amino acid profile of hoary basil seed protein

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>mg/100mg</th>
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<tbody>
<tr>
<td>Aspartic acid</td>
<td>4.61</td>
</tr>
<tr>
<td>Serine</td>
<td>3.58</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10.55</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.12</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.70</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.48</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.16</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.65</td>
</tr>
<tr>
<td>Proline</td>
<td>2.25</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.08</td>
</tr>
<tr>
<td>Valine</td>
<td>2.63</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.56</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.91</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.02</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.49</td>
</tr>
</tbody>
</table>

**Degree of hydrolysis**

The maximum DH exhibited by hoary basil seed protein hydrolysate (HBPs) in the current study (55.134 ± 0.1 %) for 270 min as show in Figure 1a. It was greater than the DH values reported for protease hydrolyzed proteins from other oilseeds such as soy, which had DH of 39.5 % upon 8 h of hydrolysis [19] and DH of 5.46 to 17.86 % upon 1 to 8 h of hydrolysis [20] and sunflower that had DH of 42.2 % upon 3 h of hydrolysis [21]. These findings are in agreement with the results of enzymatic hydrolysis of other proteins reported by other workers. The DH for rice endosperm protein [22] and wheat germ protein hydrolysates [23] was reported to be 11.7 % and 25 % after 6 h of hydrolysis respectively; the % DH increased with increase in time of hydrolysis. The maximum DH value of HBPs obtained by Protease G6 hydrolysis alone was also lower than the recently reported values for Alcalase-Flavourzyme hydrolysates of yellow pea protein hydrolysate (DH: 58.89 %), Kabuli (DH: 77.58 %) and Desi (DH: 77.53 %) chickpea protein hydrolysates prepared by the protease enzymes [24]. Hydrolyzed protein was separated into soluble supernatants, and this fraction was subjected to antioxidant assays. It was found DPPH radical scavenging ability of HBPs increased, with an increase in the degree of hydrolysis (Figure 1b).
Figure 1 (a) Hydrolysis curve of hoary basil seed protein hydrolysate (HBPs) treated with Protease G6. Reaction conditions: pH, 8.0; 50°C with shaking 150 rpm, substrate: enzyme was 0.5:10 (w/v). (b) Antioxidant activity curve for HBPs.

Antioxidant assays

Ultrafiltration membranes were used to separate the HBPs into three fractions, HBPs-I (MW > 10 kDa), HBPs-II (MW = 5-10 kDa), and HBPs-III (MW < 5 kDa). As shown in Table 2, HBPs-III (MW < 5 kDa) showed the lowest IC$_{50}$ value, which indicated the highest free radical scavenging activity. However, after ultrafiltration the in vitro antioxidant activity of the protein hydrolysate from HBPs was dramatically reduced. Previous studies support the presence as well as absence of free radical scavenging capacity activity of protein hydrolysates obtained by microbial protease catalyzed hydrolysis. Flavourzyme hydrolyzed soy concentrate upon passing through a 50 kDa membrane previously showed an activity of 21.81 % (concentration used: 0.1 mg protein/mL) at a DH of 28.8 % and this value was found to be similar to the free radical scavenging capacity activity shown by flaxseed protein hydrolysate at similar DH (25). Udenigwe et al., 2009 (26) digested flaxseed protein with pancreatin and reported that < 1 kDa ultrafiltered fraction of the resulting hydrolysate had an OH$^-$ scavenging activity with an IC$_{50}$ of 0.06 mg protein/mL. Since the protein hydrolysate from hoary basil seed showed by far the strongest antioxidation activity it was selected for further investigation, involving bioassay guided fractionation, in order to isolate the antioxidation activity constituent(s) responsible. These activities showed that this plant may have potential to be one of new effective antioxidant.

Table 2 Ultrafiltration separation of HBPs prepared by Protease G6 digestion and their free radical scavenging capacity by DPPH assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Molecular weight (kDa)</th>
<th>IC$_{50}$ (µg/ml)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBPs</td>
<td></td>
<td>14.65 ± 0.14</td>
</tr>
<tr>
<td>HBPs-I</td>
<td>&gt; 10 kDa</td>
<td>41.17 ± 0.14</td>
</tr>
<tr>
<td>HBPs-II</td>
<td>5-10 kDa</td>
<td>37.00 ± 0.13</td>
</tr>
<tr>
<td>HBPs-III</td>
<td>&lt; 5 kDa</td>
<td>25.23 ± 0.14</td>
</tr>
<tr>
<td>ascorbic acid (vitamin C)</td>
<td></td>
<td>142.80 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$All data are shown as the average mean ± 1 standard error of mean and are obtained from 3 replicated determination.
4. CONCLUSIONS

A potential HBPs-III that displays antioxidant was enriched from the hoary basil seed by an ultrafiltration procedure. HBPs-III has a high dose-dependent antioxidant activity in DPPH assays. However, determination of the antioxidant assay that plays the important roles in various diseases and aging. The mechanism(s) of such activity of HBPs-III, along with conformation of its multimeric state and role of such, await further research.

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