In vitro antioxidant of the protein hydrolysate isolated from the seeds of hoary basil (*Ocimum basilicum*)

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Abstract

Oxidation plays an important role in the basic processes of life, such as the production of energy and phagocytises employed by the immune system. However, when an imbalance between oxidants and antioxidants exists *in vivo*, oxidation can become uncontrolled and result in diseases such as arthritis, cancer, atherosclerosis, and Alzheimer's disease. The objective of this research work to produce hoary basil seed protein hydrolysates (HBPs) and their peptides with antioxidant properties. Enzymatic hydrolysis of the seeds was performed by using a bacterial alkaline protease, Protease G6 at their optimal conditions. The degree of hydrolysis of HBPs with Protease G6 hydrolysis and DPPH radical scavenging assay, IC_{50} values showed 55.134±0.10% and 0.0196±0.064 µg/mL for 270 min. These results show that HBPs increased the degree of hydrolysis and antioxidant activity of protein hydrolysates. 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 IC_{50} value at 0.02523±0.14 µg/mL for DPPH radical scavenging activity. The findings suggest the importance of hoary basil seed protein as a source of bioactive peptides.

Keywords: *hoary basil, protein hydrolysate, antioxidant activity*

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 (Berlett and Standtman, 1997). Essentially, all the cellular components and the specific constituents are susceptible to reactive oxygen species (ROS)

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and reactive nitrogen species (RNS), for example, hydroxyl radicals ($^{\cdot}OH$), peroxyl radicals ($^{\cdot}OOR$), superoxide anion ($O_2^{-\cdot}$), and peroxyl nitrite ($ONOO^{-\cdot}$). The accumulation of protein carbonyl compounds, a result of oxidation, is believed to be a main mechanism of aging process in human (Standtman 2006).

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 (Wojcik *et al*., 2010). 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 (Ryan *et al*., 2011).

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 (Lamsal *et al*., 2006). 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 (Mahmaod, 1994). 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 (Franek *et al*., 2000). 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, histedine and cysteine (Wang *et al*., 2006). These amino acids can donate proton to the free radicals resulting this hydrolysate has an antioxidant activity. Reported low molecular polypeptide components with histedine, tryptophan and tyrosine showed highly antioxidant activity. (Xie *et al*., 2008).

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 (Singh *et al*., 2013).

The initial study found that essential oil of Hoary basil composed of highly essential fatty acid (Suksai and Noitang, 2007). So hoary basil seed which is a by-product form the chemical extraction of essential oil and fiber. It is interesed material in producing active protein hydrolysate. It increas value of waste and also, also it is reported that defatted Hoary basil seed was analyzed composed of 17.16% protein (Suksai and Noitang, 2007). 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 proteaseG6 form hoary basil seed.

2. Materials and Methods

2.1 Biological and chemical materials

Hoary basil seed were purchased from Yoawarat 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), and 2, 2-Diphenyl-1-picryhydrazyl (DPPH) were purchased from Sigma Chemicals Co. (USA). Protease G6 (alkali serine protease) was purchased from Siam Victory Chemicals Co., Ltd (Thailand). All other unlabeled chemicals and reagents were of analytical grade.

2.2 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.

2.3 Total amino acid analysis

2.3.1 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.

2.3.2 Chromatographic conditions

Chromatographic separation was carried out in a Waters Alliance 2695 with heater amino acid analysis Hypersil Gold column C18. The column was thermostatted 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.

2.4 Preparation of hoary basil seed protein hydrolysate (HBPs)

The sieved hoary basil seed less than 90 meshes was hydrolyzed using ProteaseG6 (alkali serine protease), 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.

2.5 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 it was transform by Galla (Galla *et al*., 2012). Sample were treated with 10% trichloroaceticacid (TCA) and centrifuge 15,000 × g for 30 minute at 4°C. The supernatant was considered and %DH was calculated by formula below

$$DH\% = \frac{\text{Soluble Nitrogen in TCA 10\%}}{\text{Total Nitrogen in the sample}} \times 100$$

2.6 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.

2.7 DPPH radical scavenging activity

The DPPH radical scavenging activity was modulated the method of Moshen and Ammar (2009), 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 IC_{50} 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) used 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 IC_{50} values (IC_{50}: concentration required to obtain a 50% radical scavenging activity).

2.8 Protein concentration

The protein content was determined by Bradford's procedure (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard with four different concentrations between 5–20 µg/ml to construct the calibration curve.

2.9 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 and Discussion

3.1 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 (Chen *et al*., 1998). The relationship between hydrophobic amino acid and the reducing power of hydrolysates have been previously reported (Bernardini *et al*., 2012). 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 (Chen *et al*., 2012). 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 phenlyalanin, 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 (Chen *et al*., 1998). Results are comparable to those of earlier worker (Gokavi *et al*., 2004) 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

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

3.2 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 (Hrckova *et al*., 2002) and DH of 5.46 to 17.86% upon 1 to 8 h of hydrolysis (Tsou *et al*., 2010) and sunflower that had DH of 42.2% upon 3 h of hydrolysis (Villanueva *et al*., 1999). These findings are in agreement with the results of enzymatic hydrolysis of other proteins reported by other workers. The DH for rice endosperm protein (Zhang *et al*., 2009) and wheat germ protein hydrolysates (Zhu *et al*., 2006) 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 (Barbana and Boye, 2010). 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).

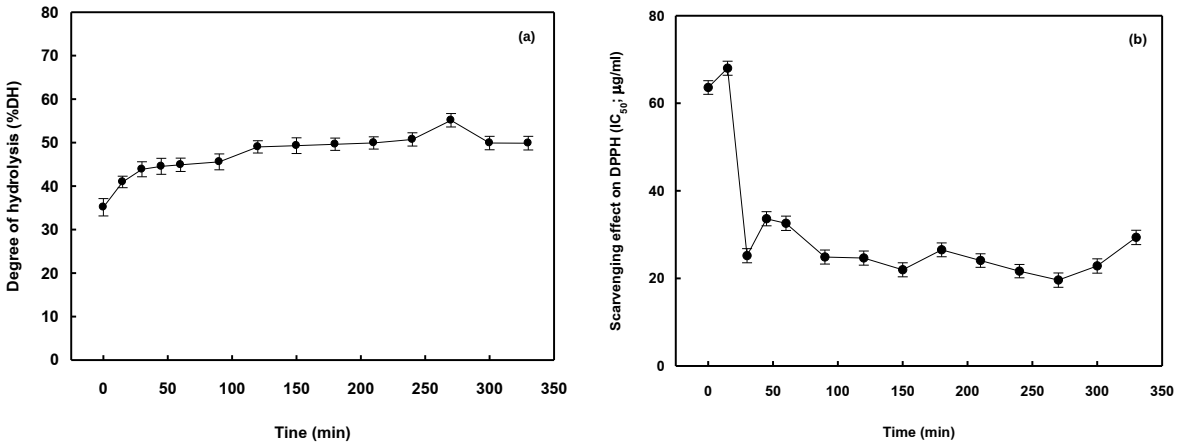

Figure 1 (a) Hydrolysis curve of hoary basil seed protein hydrolysate (HBPs) treated with Protease G6. Reaction conditions: pH, 8.0; 50°C with shaking 150 rpm., substrate : enzyme was 0.5:10 (w/v). (b) Antioxidant activity curve for HBPs.

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

Sample	Molecular weight (kDa)	IC_{50} (μg/ml)[a]
HBPs		14.65 ± 0.14
HBPs-I	>10 kDa	41.17 ± 0.14
HBPs-II	5-10 kDa	37.00 ± 0.13
HBPs-III	<5 kDa	25.23 ± 0.14
ascorbic acid (vitamin C)		142.80 ± 0.03

Note: [a] All data are shown as the average mean ± 1 standard error of mean and are obtained from 3 replicated determination.

4. Conclusion

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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References

Barbana, C., and Boye, J. I. 2010. Angiotensin I-converting enzyme inhibitory activity of chickpea and pea protein hydrolysates. Food Research International. 43: 1642–1649.

Berlett, S.S., and Stadtman, E.R. 1997. Protein oxidation in aging, disease, and oxidative stress. Journal of Biological Chemistry. 272: 20313–20316.

Bernardini, R.D., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A.M., and Hayes, M. 2011. Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. Food Chemistry. 124: 1296–1307.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254.

Chen, H.M., Muramoto, K., Yamauchi, F., Fujimoto, K., and Nokihara., K., 1998. Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. Journal of Agricultural and Food Chemistry. 46: 49–53.

Chen, N., Yang, H., Sun, Y., Niu, J. and Liu, S. 2012. Purification and identification of antioxidant peptides from walnut (*Juglans regia* L.) protein hydrolysates. Peptides. 38: 344–349.

Franek, F., Hohenwarter, O., and Katinger, H. 2000 Plant protein hydrolysate: preparation of defined peptide fractions promoting growth and production in animal cell cultures. Biotechnology Progress. 16: 688–692.

Galla, NR., Pamidighantam, PR., Akula, S., and Karabala, B. 2012. Functional properties and in vitro antioxidant activity of roe protein hydrolysates of *Channa striatus* and *Labeo rohita*. Food Chemistry. 135: 1479–1484.

Gokavi, SS., Nagappa, GM., and Guo, M. 2004. Chemical composition of garden cress (*Lepidium sativum*) seeds and its fractions and use of bran as a functional ingredient. Plant Foods for Human Nutrition 59: 105–111.

Hrckova, M., Rusnakova, M., and Zemonovic, J. 2002. Enzymatic hydrolysis of defatted soy flour by three different proteases and their effect on the functional properties of resulting protein hydrolysates. Czech Journal of Food Sciences. 20: 7–14.

Lamsal, B.P., Murphy, P.A., and Johnson, L.A. 2006. Flaking and extrusion as mechanical treatments for enzyme-assisted aqueous extraction of oil from soybeans. Journal of American Oil Chemists Society. 83: 973–979.

Mahmoud, M.I., 1994. Physicochemical and functional properties of hydrolysates in nutritional products. Food Technology 48: 89–95.

Mohsen, S.M., and Ammar, A.S.M. 2009. Total phenolic contents and antioxidant activity of corn tassel extracts. Food Chemistry. 112: 595–598.

Moure, A., Dominguez, H. and Parajo, J.C. 2006. Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. Process Biochemistry 41: 447–456.

Ryan, J.T., Ross, R.P., Bolton, D., Fitzgerald G.F., and Stanton, C. 2011. Bioactive peptides from muscle sources: Meat and Fish. Nutrients. 3: 765–791.

Singh, R., Soni, S., Patel, R., and Kalra, A. 2013. Technology for improving essential oil yield of *Ocimum basilicum* L. (sweet basil) by application of bioinooculant colonized seeds under organic field condition. Industrial Crops and Products. 45: 335–342.

Standtman E.R. 2006. Protein oxidation and aging. Free Radical Research. 40: 1250–1258.

Suksai, S., and Noitang, S. 2007. Extraction of oil from Hairy basil (*Ocimum spp*) seeds and swelling properties of mucilage from residues. Research reports: Chulalongkorn UniversityIntellectual Repository. [Online] http://hdl.handle.net/123456789/8711

Tsou, M.J., Kao, F.J., Tseng, C.K., and Chiang, W.D. 2010. Enhancing the antiadipogenic activity of soy protein by limited hydrolysis with Flavourzyme and ultrafiltration. Food Chemistry. 122: 243–248.

Udenigwe, C.C., Lu, Y.L., Han, C.H., Hou, W.C., and Aluko, R. E. 2009. Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. Food Chemistry. 116: 277–284.

Villanueva, A., Vioque, J., Sánchez-Vioque, R., Clemente, A., Pedroche, J., Bautista, J., and Millán, F. 1999. Peptide characteristics of sunflower protein hydrolysates. Journal of American Oil Chemists' Society. 76: 1455–1460.

Wang, Y.C., Yu, R.C., and Chou, C.C. 2006. Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. Food Microbiology. 23:128–135.

Wojcik, M., Burzynska-Pedziwiatr, I., and Wozniak, L.A. 2010. A review of natural and synthetic antioxidants important for health and longevity. Current Medicinal Chemistry. 17: 3262–3288.

Xie, Z., Huang, J., Xu, X., and Jin, Z. 2008. Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. Food Chemistry. 111: 370–376.

Zhu, K., Hui-Ming, Z., and Hai-Feng, Q. 2006. Proteins extracted from defatted wheat germ: Nutritional and structural properties. Cereal Chemistry. 83: 69–75.