In vitro anti-tyrosinase activity of protein hydrolysate from spotted babylon (Babylonia areolata)

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Abstract

Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis. Melanin plays an important role in protecting human skin from the harmful effects of ultra violet radiation. Tyrosinase is the key enzyme in melanin biosynthesis. In case of over-activity can lead to dermatological disorder. The aim of this study was to investigate new tyrosinase inhibitors of protein hydrolysate from spotted babylon. Protein hydrolysate was fractionated by ultrafiltration, MW < 5,000 Da showed the potency as tyrosinase inhibitor with IC_{50} value of 0.075±0.004 µg/ml using L-DOPA as substrate which are strong about four times than the positive control kojic acid (IC_{50} = 0.251±0.007 µg/ml). The tyrosinase inhibitory activity effect from retentate of 5 kDa membrane of spotted babylon protein hydrolysate was moderately thermostable (showed maximum tyrosinase inhibitory activity about 90°C with 68% for 30 min), showed <50% activity across a broad pH range of 3–12. In order to know the compounds responsible for tyrosinase inhibitory activities, the identification and characterization of protein hydrolysates of spotted babylon are currently underway in our laboratory. The search for more potent tyrosinase inhibitors from protein hydrolysate are still needed for the treatment of various dermatological disorders and are also applied as cosmetic whitening agents.

Keywords: tyrosinase inhibitor, protein hydrolysate, spotted babylon

Introduction

The color of mammalian skin and hair is determined by a number of factors, the most important of which is the degree and distribution of melanin pigmentation. Melanin is one of the most widely distributed pigments and is found in bacteria, fungi, plants and animals. In animals, it is secreted by melanocyte cells distributed in the basal layer of the dermis (Kim

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and Uyama, 2005). It is a heterogeneous, polyphenol-like biopolymer with a complex structure and color varying from yellow to black (Choi *et al*., 2006). The control of melanogenesis is an important strategy in the treatment of abnormal skin pigmentation for cosmetic purposes (Im *et al*., 2002). Melanin synthesis is mainly controlled by tyrosinase, a copper-containing enzyme that catalyzes two distinct reactions in melanin synthesis, via the hydroxylation of tyrosine by monophenolase action and the oxidation of 3, 4-dihydroxy-L-phenylalanine (L-DOPA) to o-dopaquinone by diphenolase action (Song *et al*., 2006). o-dopaquinone is unstable in aqueous solution and rapidly suffers a non-enzymatic cyclization to leukodopachrome, which is further oxidized non-enzymatically by another molecule of o-dopaquinone to yield dopachrome and one molecule of regenerated L-DOPA (Cooksey *et al*., 1997). The modulators of melanogenesis can act directly on tyrosinase activity, which is responsible for the conversion of melanosomes to keratinocytes (Kim *et al*., 2006). It has been reported that transcription factors such as lymphoid-enhancing factor-1 are involved in the expression of tyrosinase-related proteins such as TRP-1 and TRP-2 (Sato and Toriyama, 2009).

Hydrolysate protein is a product of protein digestion by cutting the polypeptide to the free amino acids or short peptides using chemical or enzymatic hydrolysis reaction. Source of hydrolysate protein for most of the materials are cheap (Mahmaod, 1994). Enzyme hydrolysis is widely used because chemical hydrolysis is conducted under strong chemicals and solvents at extreme conditions and providing yields product with reduced nutritional quality (Kristinsson *et al*., 2000). Therefore, tyrosinase inhibition by proteins and protein hydrolysates as well as individual peptides and amino acids has been investigated. Proteins and peptides from natural resources such as milk (Chen *et al*., 2006; Nakajima *et al*., 1996), wheat (Okot-Kotber *et al*., 2001), honey (Oszmianski *et al*., 1990; Ates *et al*., 1990), silk (Kato *et al*., 1998), and the housefly (Daquinag *et al*., 1995; Daquinag et al., 1999) appeared to be able to inhibit tyrosinase activity. Other tyrosinase inhibitory peptides investigated are cyclic peptides (Morita *et al*., 1994), kojic acid-tripeptides (Kim *et al*., 2004), and dipeptides (Girelli *et al*., 2004). Spotted Babylon is belonging to the family Buccinidae, genus *Babylonia* is an important gastropod consumption in Thailand. Spotted babylon have a highly nutritional value consisted of protein 18.78%, fat 2.86%, carbohydrate 5.18%, ash 5.27%, and moisture 67.91%. Moreover, the nutrition values from a small size spotted babylon (shell length 3-4cm) and a big size (shell length 5-7cm) is not difference (Chaitanavisuti *et al*., 2013). This research, therefore, recognizes spotted babylon might be the important source of protein hydrolysate. The objective of this research was to determine tyrosinase inhibitory properties of protein hydrolysate obtained from spotted babylon produced by Protease G6. The tyrosinase inhibitory fraction

from the spotted babylon protein hydrolysate could be an alternative for recent commercial product.

2. Materials and Methods

2.1 Biological and chemical materials

Spotted babylon was captered from a farm at Sichang Marine Science Research and Training Station, Chulalongkorn University, located on Sichang Island Cholburi province, Thailand. The samples were quickly taken to the laboratory and kept in the desiccator until used. Bovine serum albumin (BSA), L-DOPA, kojic acid, and tyrosinase from mushroom were purchased from Sigma Chemicals Co. (USA). Protease G6 (alcalase) was purchased from Siam Victory Chemicals Co., Ltd (Thailand). All other chemicals used were of analytical grade.

2.2 Preparation of spotted babylon powder

Spotted babylon were first anaesthetized with ice. The muscle was separated and blended using a mixer. The homogenate was then defatted according to the method described Wang et al. (Wang *et al*., 2013) by using isopropanol at a ratio of 1:4 (w/v) with continuous stirring for 1 hour at room temperature. Isopropanol was then removing from the homogenate by vacuum filtration and dried at 60°C. Dried homogenate was milled into a powder, and referred to as spotted babylon powder.

2.3 Total amino acid analysis

2.3.1 Acid hydrolysis

Five milliliters of HCl 6 N 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 2.5mM 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

Liquid chromatography was performed using a Waters Alliance 2695 separation module (Waters, Milford, MA, US). Analytes were separated on a Hypersil Gold column C_{18} column (Waters), eluted isocratically with sodium acetate buffer pH 4.90 and 60% acetonitrile,

delivered at a flow rate of 0.3 ml/min. The injection volume was 5 µl. The total run time of the method was 15 min.

2.4 Preparation of protein hydrolysate

Spotted babylon powder was hydrolysed using Protease G6 at 5 concentration in a ratio 1:1 (5.8×10^5 DU/g), 1:2 (2.9×10^5 DU/g), 1:4 (1.45×10^5 DU/g), 1:8 (7.25×10^5 DU/g), 1:16 (3.625×10^5 DU/g)(w/v) in 20 mM Tris-HCl buffer containing 150 mM NaCl pH 8.0 and using a substrate : enzyme ratio of 0.5:10 (w/v). The hydrolysis was conducted at 50°C in a shaking waterbath at 150 rpm for 4 hours. The hydrolysis reaction was stopped by heating at 90°C for 10 min. After that, the reaction mixture was centrifuged at 15,000×g for 15 min at 4°C and the supernatant was collected. The protein content of the supernatant was determined by Bradford's procedure.

2.5 Ultrafiltration

The protein hydrolysates were fractionated by ultrafiltration membranes using a bioreactor system (Amersham Biosciences, Sweden). Spotted babylon protein hydrolysate 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. Three fractions were collected from the membrane filtration including retentate from 10kDa membrane, retentate from 5 kDa membrane and permeate from 5 kDa membrane.

2.6 Tyrosinase inhibition assay

Tyrosinase inhibition assay was slightly modified from Batubara *et al.*, 2010. 35 µl of each protein hydrolysate fraction was combined with 15 µl of tyrosinase (333 U/ml in 20 phosphate buffer 50 mM pH 6.5) and incubated at 25°C for 5 min. 55 µl of substrate (L-DOPA 12 mM) was then added and incubated at 25°C for 30 min. The determination was done in triplicate. The absorbance measuring at 510 nm was determined by microplate reader. Kojic acid was used as positive control. The percent inhibition of tyrosinase was calculated as follows:

$$\% \text{ Inhibition} = \frac{[\text{Abs control} - (\text{Abs Sample} - \text{Abs Background})] \times 100}{\text{Abs control} - \text{Abs Blank}}$$

Where *Abs*Control is the absorbance of control (no sample),

*Abs*Sample is the absorbance of sample,

*Abs*Background is the absorbance of background (color of sample)

and *Abs*Blank is the absorbance of blank (deionized water).

The IC_{50} was the concentration of protein hydrolysate where 50% of enzyme activity was inhibited.

2.7 Effect of temperature on the tyrosinase inhibitory activity

The effect of temperature on the tyrosinase inhibitory activity was investigated according to the method described by Rungsaeng *et al*., 2013 with a slight modification. The protein hydrolysate was dissolved in 20 mM phosphate buffer pH 7.2 and incubated at various temperature (-20 to 90°C) for 30, 60, 90, and 120 min follow by tyrosinase inhibition assay. The result of tyrosinase inhibition assay was compared with control (no sample) which was defined as 100% and then reported as the relative activities compared to the control.

2.8 Effect of pH dependence of tyrosinase inhibitor activity

Protein hydrolysate was incubated in buffer at various pH ranging from 2–12 according to the method described by Rungsaeng *et al*., (2013) to investigate the pH stability and the pH optimal of tyrosinase inhibitory activity. All buffers used (20 mM) were consisted of glycine-HCl (pH 2–4), sodium acetate (pH 4–6), potassium phosphate (pH 6–8), Tris-HCl (pH 8–10), and glycine-NaOH (pH 10–12). The protein hydrolysate was combined in the difference buffer-pH, incubated for 30, 60, 90, and 120 min before the tyrosinase inhibition assay at room temperature. The result of tyrosinase inhibition assay were compared to the control (set as 100%) and then reported as the % relative activity.

2.9 Protein concentration assay

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.

2.10 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 calculate 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 spotted babylon powder

Amino acid composition and content of spotted babylon are shown in Table 1. The spotted babylon comprised of aspartic acid, serine, glutamic acid, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, lysine, isoleucine, leucine and phenylalanine at difference content. Spotted babylon powder contained 1.66 mg/100mg of tyrosine. Because of the tyrosine as substrate in the melanin synthesis, therefore it should not have a large amount of tyrosine in protein hydrolysate. Because it might increase substrate concentration and reduce the inhibitory activity. Schurink *et al.*, (2007) described that good tyrosinase inhibitory peptides consisted of arginine residues and/or phenylalanine. Moreover, in the presence of hydrophobic amino acids, aliphatic amino acids such as valine, alanine, or leucine is considered more important in the inhibition of tyrosinase activity, because they might inhibit quinone formation. From the Table 1 indicated that spotted babylon contain high amounts of arginine which plays a crucial role in inhibition of tyrosinase.

Table 1 Total amino acid profile of spotted babylon protein

Amino acids	mg/100mg
Aspartic acid	6.16
Serine	3.00
Glutamic acid	10.36
Glycine	4.62
Histidine	0.96
Arginine	6.38
Threonine	2.95
Alanine	4.06
Proline	2.92
Tyrosine	1.66
Valine	2.70
Lysine	4.15
Isoleucine	2.24
Leucine	4.98
Phenylalanine	2.01

3.2 Optimization of enzymatic hydrolysis condition of spotted babylon by Protease G6

The IC_{50} calculated by the regression equation obtained from evaluation of enzymatic hydrolysis condition by Protease G6 with different concentrations and times interval has been presented in Figure 1. Of five difference enzyme concentration screened, all gave tyrosinase inhibitory activity positive with good inhibitory activities (low IC_{50} values) that were observed for concentration 1:1 (5.8×10^5 DU/g) (w/v) at 210 min (0.026±0.002 µg/ml), and it was thus selected for further enrichment of the tyrosinase inhibitory and evaluation.

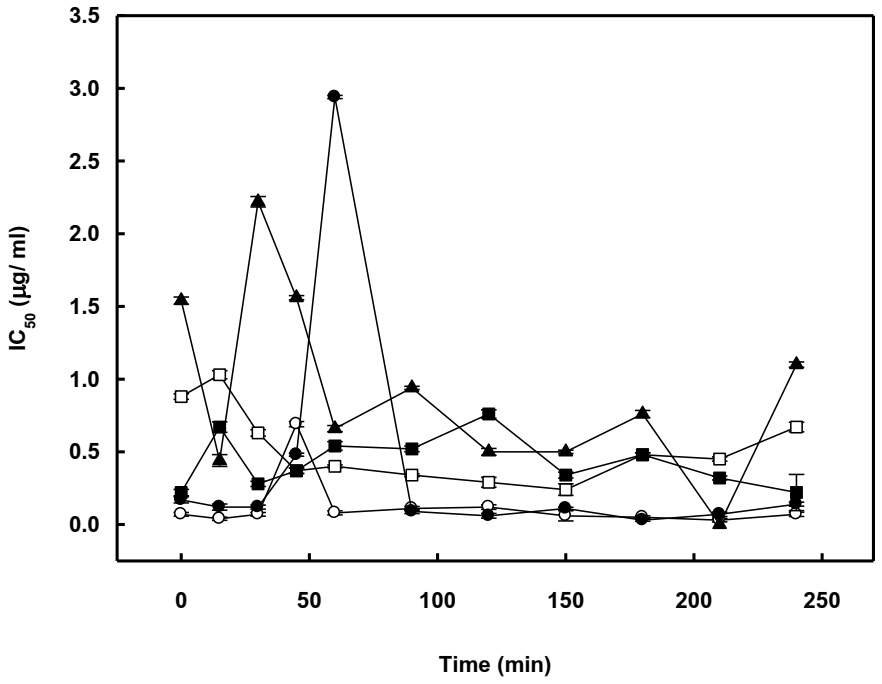

Figure 1 The IC_{50} value of protein hydrolysate at difference enzyme concentration in a ratio (○; 1:1, ●; 1:2, □; 1:4, ■; 1:8, and ▲; 1:6) at various time. Data are shown as the mean ± 1 SEM and are derived from triplicate experiments.

3.3 Inhibitory effect of protein hydrolysate fraction on tyrosinase activity

The hydrolysate was further fractionated into peptide sizes of MW>10 kDa, 5kDa<MW<10kDa and MW<5kDa using membrane ultrafiltration. The membrane ultrafiltration fractions were assayed for *in vitro* tyrosinase inhibitory activities. In addition, the 50% inhibition value (IC_{50} value) for MW<5kDa showed the lowest IC_{50} value (0.075±0.004 µg/ml) was significantly (3.34-fold) lower than that of the commonly used synthetic tyrosinase inhibitor, kojic acid (IC_{50} values of 0.251±0.007µg/ml). Moreover, Manosroi *et al.*, (2010) reported that all sericin samples showed tyrosinase inhibition activity with IC_{50} values in the range of 1.20–18.76 mg/ml which were lower activity than our result (MW<5kDa). In order to know the

compounds responsible for tyrosinase inhibitory activities, the identification and characterization of protein hydrolysate of spotted babylon are currently underway in our laboratory.

Table 2 Ultrafiltration separation of spotted babylon protein hydrolysates prepared by Protease G6 hydrolysis and their tyrosinase inhibiton activity assay

Molecular weight (kDa)	Inhibition (%)	IC_{50} (μg/ml)[a]
＞ 10 kDa	60.69±0.006	0.378±0.006
5–10 kDa	57.60±0.005	0.263±0.005
＜ 5 kDa	62.84±0.003	0.075±0.004
Kojic acid	90.14±0.007	0.251±0.007

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

3.4 Temperature and pH resistance determination

The thermal stability profile of the tyrosinase inhibitory activity from retentate of 5 kDa membrane of spotted babylon protein hydrolysate is shown in Figure 2 (a). The result showed maximum tyrosinase inhibitory activity at 90°C with 68% for 30 min. The higher temperature and longer incubation time range may have caused a change in the protein structure at regions that are involved in binding to tyrosinase. The residual tyrosinase inhibitor activity, reported as a relative percent inhibition, as a function of the pH was largely affected, giving a broad pH range of activity from 3 to 12. However, some buffer-dependent affects were seen, especially at pH 4.0 where a very low tyrosinase inhibitory activity was seen in glycine-HCl but not in sodium acetate. Thus, some inhibitor-ion interactions might block or slow down the tyrosinase inhibitory activity at such pH values. These are potential pitfalls in all, including this tyrosinase inhibitory activity, enzyme assays and also in potential biotechnological applications where changing buffers is difficult or expensive.

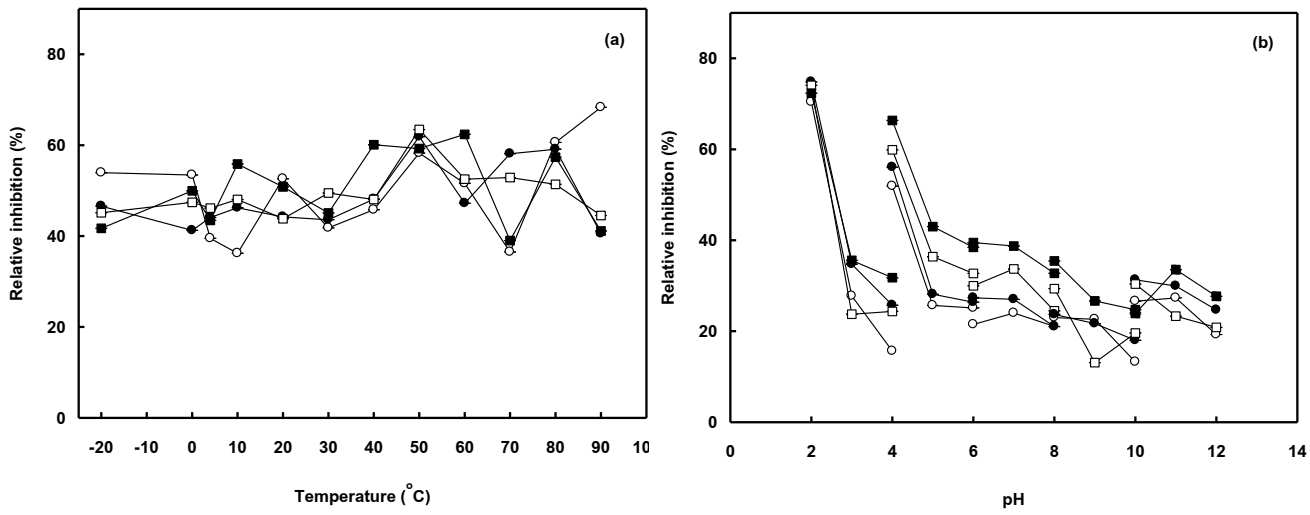

Figure 2 (a) Thermostability of the tyrosinase inhibitory activity from the retentate of 5 kDa membrane of spotted babylon protein hydrolysate. The assay was performed in 20 phosphate buffer 50 mM pH 6.5 at various temperatures for (○) 30, (●) 60, (□) 90 and 120 (■) min).

(b) pH stability of the tyrosinase inhibitory from the retentate from 5 kDa membrane of spotted babylon protein hydrolysate. The assay was performed in the following buffer systems (all 20 mM); glycine-HCl (pH 2.0-4.0), sodium acetate (pH 4.0-6.0), potassium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-10.0) and glycine-NaOH (pH 10.0-12.0) at various temperatures for (○) 30, (●) 60, (□) 90 and 120 (■) min. Data are shown as the mean ± 1 SEM and are derived from triplicate experiments.

4. Conclusion

The *in vitro* tyrosinase inhibitory activity of the MW<5kDa from spotted babylon was significantly higher than tyrosinase inhibitor, kojic acid. The findings from the present study suggested that enzymatic hydrolysis of spotted babylon combined to ultrafiltration fractionation of hydrolysate could provide new opportunities indicated to be a potential material for the treatment of difference disease.

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