

Original Research Article

Effect of Purified Protein Fractions from Soybean on Tyrosinase Inhibition

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Abstract: Skin is an important barrier that protects the body from damage due to its direct contact with the outside environment. Skin has epidermal units that are responsible for melanin production and distribution, a process called melanogenesis that is regulated by various enzymes, including tyrosinase. Due to its central role in melanogenesis, tyrosinase is a key target for screening and discovery of new inhibitors. The aim of this study was to investigate the efficacy of purified protein fractions from soybean (*Glycine max*) on inhibiting tyrosinase *in vitro*. Protein was extracted by using isoprecipitation method, followed by protein purification using Fast Protein Liquid Chromatography (FPLC) with DEAE anion exchange system and gradient NaCl elution. Among 9 purified protein fractions, there were 2 protein fractions (10 µg/mL) with molecular weights of 42 and 46 kDa exerted significant inhibition up to 95% on tyrosinase activity *in vitro*. In conclusion, purified protein fraction from soybean demonstrated potential tyrosinase inhibitory effect, indicating their potential use on cosmeceutical application.

Keywords: tyrosinase inhibition, soybean, *Glycine max*, purified protein fractions, Fast Protein Liquid Chromatography

INTRODUCTION

The appearance of the skin is broadly associated with beauty. Therefore, the desire to have a skin looking healthy and free of spots is the goal of many individuals, especially women. Skin pigmentation disorders consist of an over production or uneven distribution of melanin [1]. Melanin is the main component determining the color of skin and up to 10% of cells in the inner most layer of the epidermis produce melanin pigments. Melanin biosynthesis, or melanogenesis, is a wellknown physiological response of human skin upon exposure to ultraviolet light and other stimuli. Melanogenesis is regulated by enzymes, such as tyrosinase, tyrosinase related protein-1 (TRP-1), and TRP-2 [2].

Tyrosinase is an organometallic enzyme that contains copper cations, functioning as an important oxidizing agent. This enzyme is responsible for production of melanin, an important biological pigment for protection against ultraviolet irradiation and responsible for the color of skin [3]. Tyrosinase catalyzes three steps in melanin biosynthesis, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to dopaquinone and the oxidation of 5,6-

dihydroxyindole (DHI) to indolequinone [4]. Due to its central role in melanogenesis, tyrosinase is a key target for the screening and the discovery of new inhibitors.

Tyrosinase inhibitor makes melanin production diminishing, because the activity of this enzyme is the ratecontrolling step of melanin synthesis [5]. The tyrosinase inhibitors could be classified into four types, including competitive inhibitors, uncompetitive inhibitors, mixed type (competitive/uncompetitive) inhibitors, and noncompetitive inhibitors. A competitive inhibitor combines with a free tyrosinase that prevents substrate binding. A competitive inhibitor might be a copper ion chelator, tyrosinase substrate analogs, or derivatives of L-tyrosine or L-DOPA. On the other hand, an uncompetitive inhibitor only binds to the tyrosinase substrate complex. A mixed (competitive and uncompetitive mixed) type inhibitor binds not only with a free tyrosinase, but also with the tyrosinase substrate complex. For most mixed type inhibitors, their equilibrium binding constants for the free tyrosinase and the tyrosinase substrate complex are different. The non-competitive inhibitors could bind to a free tyrosinase and a tyrosinase substrate complex, with the same equilibrium constant [6]. Tyrosinase inhibitors are

useful in the cosmetic industry because of their skin whitening effects, as well as in the food industry because of their ability to inhibit the enzymatic browning of food products [7].

Soybean belongs to the family *Leguminosae*, sub family *Papilionoideae*, and the genus *Glycine* L. The cultivated form, *G. max* L. Merrill, grows annually and its plant is bushy with height ranging from 0,75 to 1,25 m, branching sparsely or densely, depending on cultivars and growing conditions [8]. Soybean proteins have been widely applied in food products, due to their nutrition values and abilities to improve food texture [9]. The analysis by SDS-PAGE confirmed the presence of purified 2S fraction (MW 20 kDa), α (MW 63,17 kDa), α' (MW 58,06 kDa) and β (MW 42,09 kDa) sub units of the 7S fraction and the acidic (MW 38,8 kDa) and basic (MW 21,04 kDa) sub units of the 11S fraction of *G. max* [10]. The aim of this study was to investigate the efficacy of purified protein fractions from soybean on inhibiting tyrosinase activity *in vitro*.

MATERIALS AND METHODS

Extraction and purification of protein from soybean

Dried soybean (*G. max*) was purchased from a traditional market in Jakarta (Indonesia). Protein extraction was done by using isoelectric precipitation [11]. Dried soybean were blended to obtain powder. A 25 g of powder was dissolved in 100 mL aquadest and mixed for 15 min. The solution was added with NaOH 1 M to increase pH value up to 8,6, and centrifugation was done at 5000 \times g for 30 min at 4°C to obtain the supernatant. Furthermore, HCl was added to supernatant to decrease pH value (4,5) and centrifugation was done at 1500 \times g for 20 min at 4°C to obtain the protein pellet. The pellet was freeze-dried for 18 h to produce protein crude.

For protein purification, a 5 g of protein crude was dissolved in 100 mL phosphate buffer 50 mM and mixed for 15 min followed by centrifugation at 5000 \times g for 15 min at 4°C. A 25 mL of supernatant was dissolved in 75 mL acetone and incubated in the refrigerator for 18 h, followed by freeze drying for 6 hours to obtain protein precipitate. Purification of protein was done by using Fast Protein Liquid Chromatography (FPLC) with employing DEAE HiTrap column as an anion exchange system and NaCl 1 M in 50 mM phosphate buffer (pH 6,5) as eluent. Flow rate was set up at 1 mL/min. All fractions were collected for further protein characterization.

Determination of protein concentration

Protein concentration was measured according to the Bradford method [12]. Protein solution containing 10 to 100 μ g/mL in a volume up to 0,4 mL was pipetted into test tubes. The volume of the test tubes was adjusted to 0.4 mL with the appropriate buffer. A 8 mL of protein

reagent was added to the test tube and the content was mixed, followed by measuring the absorbance at 595 nm. Aquadest was used as negative control and bovine serum albumine was used as a positive control. Experiments were done in triplicate. Standard curve was prepared to calculate protein concentration.

Determination of molecular weight

Molecular weight of protein were measured with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using 12% resolving gel and 5% stacking gel [13]. A 100 mg of protein was dissolved in sample buffer, 1:1 (v/v) and heated for 5 minutes at 95 °C, followed by centrifugation at 3000 \times g for 15 minutes at 4°C to obtain supernatant. A 15 μ L sample was loaded into the well, and the electrophoresis was run at 100 voltage for 150 minutes. The gel was stained with 1% w/v Coomassie Brilliant Blue R-250 (in 450 mL methanol, 450 mL aquadest, and 100 mL glacial acetic acid), and destained in 10% v/v acetic acid (methanol:acetic acid:aquades, 20:10:80 (v/v/v)). Low molecular weight (LMW) was used as protein marker. A standard curve of the log (molecular weight) versus Rf was generated to determine molecular weight of protein.

Assay of tyrosinase inhibition

Tyrosinase inhibitory activity was measured using a modified method of Yamauchi *et al.* [5]. Protein sample (10-100 μ g/mL) were diluted in 25% v/v dimethyl sulfoxide, tyrosinase (333 U/mL in 50 mM PBS pH 6.5), and fresh L-tyrosine-3,4-dihydroxy-L-phenylalanine (L-DOPA) 12 mM. A 70 μ L protein sample, 30 μ L tyrosinase 333 U/mL, and 110 μ L (L-DOPA) 12 mM were loaded into a 96-well plate. After incubation at 37°C for 30 minutes, the absorbance (A) was measured using microplate reader at 515 nm. Ascorbic acid was a positive control and aquadest as a negative control. Experiments were done in triplicate. The percentage of tyrosinase inhibition was calculated as follow: Percentage tyrosinase inhibition (%) = $100 - [(A_{\text{sample}} - A_{\text{control}}) / (A_{\text{control}})] \times 100\%$.

Statistical analysis

Data were expressed by computational analysis (SPSS 12.0), and the significance of the differences was assessed via a *t*-test. A value of $p < 0.05$ was taken as statistically significant.

RESULTS AND DISCUSSION

Extraction and purification of protein

Our results showed that protein yield of soybean was 23.6% and protein concentration was 6,10 μ g/mL. Proteins are polymer of amino acids and their relative proportion represents its quality that is dependent on the genetic make up of legumes. The variations protein contents are attributed to genetic make up legumes along with some environmental factors. Igbal *et al.*

demonstrated that the approximate protein contents of 3 major legumes per 100 g as follow: chickpea (24%), lentil (26,1%), and green pea (24,9%) [14]. Previous study by Gaur *et al.* demonstrated that chickpea (*Cicer arietinum*) contained high protein (~20%) [15]. Meanwhile, protein contents in hazelnut (*Corylus avellana*) and macademia nut (*Macadamia integrifolia*) were found to be approximately 13,7 and 7,9 g per 100 g of nuts [16].

Further study on protein purification from soybean was done by employing anion exchange system using DEAE HiTrap column and gradient NaCl

elution. Protein crude (5% w/v) from soybean was initially precipitated using acetone to obtain protein precipitate (0.3 g) used for further fractionation using FPLC system. Chromatogram profile showed that there were 3 protein peaks with 9 protein fractions (the 1st peak was A6, A7, A8 and A9, the 2nd peak was B11 and B12, and the 3rd peak was B6, B7 and B8) had been collected (Figure 1). However, only the 3rd peak containing protein fractions of B6, B7, and B8 showed protein concentration, i.e. B6 (0, 80 µg/mL), B7 (1,50 µg/mL), and B8 (1,50 µg/mL). Thus, these fractions were used for further protein characterization and tyrosinase inhibitory assay.

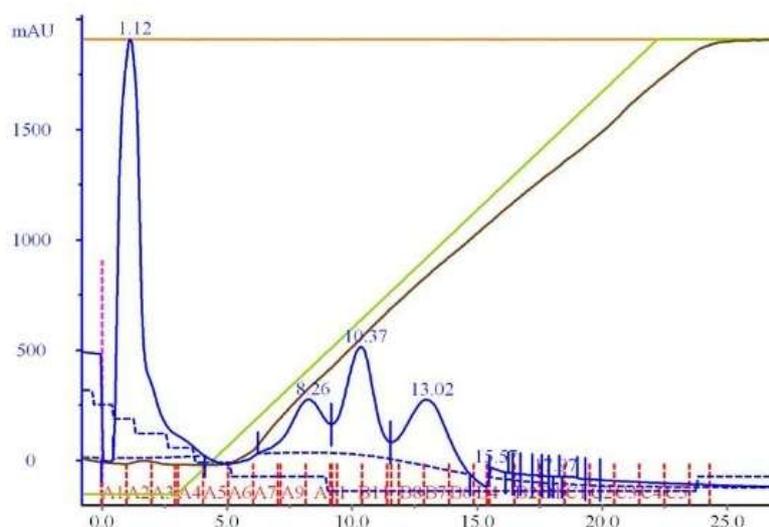


Fig-1: FPLC chromatogram profile of protein precipitate from soybean (*G. max*) using DEAE HiTrap with flow rate of 1 mL/min and gradient NaCl elution (0-1 M) in 50 mM phosphate buffer (pH 6,5)

Protein profile by SDS-PAGE

SDS-PAGE profile revealed that crude and precipitate proteins (CP, PP) from soybean consisted of 3-4 distinct protein bands with approximately molecular weights of 14-67 kDa (Figure 2). Among 3 purified

protein fractions, only B7 and B8 fractions showed 2 protein bands with molecular weights of 42 and 46 kDa. In SDS-PAGE analysis, high purified protein is characterized by single protein band with specific molecular weight [17].

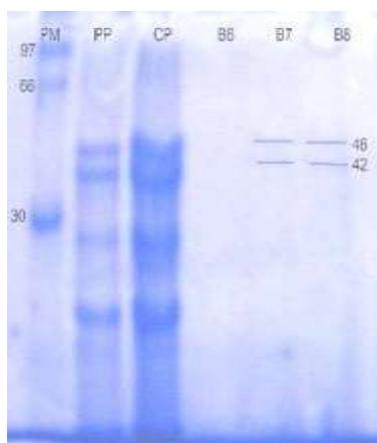


Fig-2: SDS-PAGE profile of crude (CP), precipitate (PP), and purified protein fractions (B6, B7, and B8) from soybean

Similar study by Kumar *et al.* and Leite *et al.* demonstrated that soybean protein fractions from *G. max* seed consisted of 2S fraction (20 kDa), α (63,17 kDa), α' (58,06 kDa), and β (42,09 kDa) sub units of the 7S fraction, the acidic (38,8 kDa) and basic (21,04 kDa) sub units of the 11S fraction [10, 18]. It has been known that the majority of soybean protein is globulin that was grouped in glycinin and conglycinin [8]. Kunte *et al.* showed that soybean consisted of 2 soy globulin fractions of 7S and 11S [19]. Other study reported that soybean 7S protein and β -conglycinin have been isolated and identified, and β -conglycinin is composed of 3 protein sub units, namely α , α' , and β [20].

The β -conglycinin has high contents of alanine, proline, leucine, valine and isoleucine; while glycinin contains high isoleucine, proline, leucine, valine, and phenylalanine [21]. Keshavarz and Nakai reported that in addition to the hydrophobic amino acids, the nonpolar portion of the amino acid lysine

participates in interactions that are essential in gel formation [22]. Various composition of hydrophobic amino acids, such as isoleucine, proline, phenylalanine, leucine, and valine may be the primary cause of differences in functionality and physical characteristics of soybean proteins.

Tyrosinase inhibitory activity

We tested the efficacy of soybean protein and its purified fractions on inhibiting tyrosinase activity *in vitro*. Figure 3 demonstrated that all soybean proteins in crude (CP), precipitate (PP), and purified fractions (B6, B7, and B8) exerted significantly inhibitory activities on tyrosinase >50%, respectively. Among 3 purified fractions, only B7 and B8 fractions demonstrated significant inhibition on tyrosinase. Interestingly, purified fractions of B7 and B8 had similar efficacy with standard ascorbic acid. Thus, we may apply these proteins in development and formulation of various products using crude, precipitate, and purified fractions.

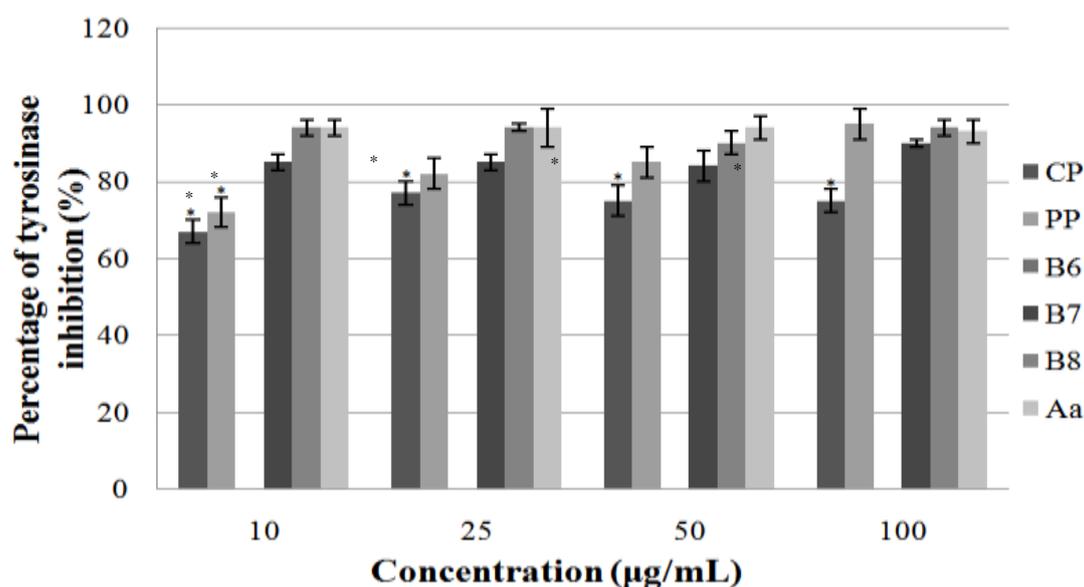


Fig-3: Effect of crude (CP), precipitate (PP), and purified protein fractions (B6, B7, B8) at various doses (10-100 µg/mL) on inhibition of tyrosinase activity *in vitro*. Ascorbic acid (Aa) was used as a standard reference. * $p < 0.05$ against Aa

Tyrosinase inhibitors from natural sources have great potential, as they are considered to be safe and largely free from adverse side effects. 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, wheat, honey, silk, and the housefly appeared to be able to inhibit tyrosinase activity. Other tyrosinase inhibitory peptides investigated are cyclic peptides [23]. In terms of its potential as tyrosinase inhibitor agent, Liang *et al.* and Cho *et al.* demonstrated that proteins from black soybean sprout and brown soybean exerted significant

tyrosinase inhibition >90%, respectively [7, 24]. These data are in line with our results (Figure 3). Some peptides also showed a concentration dependent reduction in tyrosinase activity. Peptide from kefir whey at 15 mg/mL inhibit tyrosinase up to 50% [25]. Tripeptide amides synthesis (phe, trp, and tyr) inhibit tyrosinase ~92% [26].

CONCLUSION

Purified protein fractions with molecular weights of 42 and 46 kDa have been extracted from soybean extract. Both purified protein fractions demonstrated significant and potential inhibitory effect

on tyrosinase activity *in vitro*, indicating their potential use on cosmeceutical application.

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