J Appl Biotechnol Rep. 2026 June;13(2):2035-2043

Journal of
Applied Biotechnology Reports

doi 10.30491/jabr.2025.531906.1888

Original Article

Tyrosinase Inhibitory Activity of Black Soldier Fly (*Hermetia illucens*) Larval Hydrolysate

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Received July 2, 2025; **Accepted** November 22, 2025; **Online Published** June 30, 2026

Abstract

Introduction: Black Soldier Fly (BSF) (*Hermetia illucens*) larvae contain protein in varying amounts depending on the age at harvest. Due to their high protein content, BSF larvae represent a promising alternative protein source with potential bioactivities, including tyrosinase inhibitory activity. This study aimed to evaluate the tyrosinase inhibitory activity of BSF larval protein hydrolysates as a function of larval age and the fractionation process.

Materials and Methods: The larvae were dried and defatted using the Soxhlet extraction method. The defatted larvae were characterized by determining ash, moisture, and soluble protein contents. Enzymatic hydrolysis was carried out using Alcalase®. Soluble protein content in the hydrolysates was analyzed using the Bradford assay. The degree of hydrolysis (DH) of the hydrolysates was analyzed using the o-phthaldialdehyde (OPA) method. The hydrolysates were then freeze-dried and fractionated using C18 Solid Phase Extraction (SPE) before tyrosinase inhibition assays.

Results: The highest tyrosinase inhibitory activity among the samples was observed in the 15-day-old (D15 larvae) hydrolysate at a concentration of 40,000 ppm, showing 88.98% inhibition. In comparison, inhibition at the same concentration was 75.77% for 5-day-old (D5 larvae), 82.07% for 10-day-old (D10 larvae), and 87.78% for 13-day-old (D13 larvae) hydrolysates.

Conclusions: BSF larval protein hydrolysates exhibit varying levels of soluble protein depending on larval age. Moreover, the C18 SPE fractionation process yielded a higher percentage and more uniformity of tyrosinase inhibition than the unfractionated sample.

Keywords: *Hermetia illucens,* Tyrosinase Inhibitory, Enzymatic Hydrolysis, Solid-phase Extraction, Larval hydrolysate

Citation: Pramastya H, Teng FS, Rizaldy D. Tyrosinase Inhibitory Activity of Black Soldier Fly (*Hermetia illucens*) Larval Hydrolysate. J Appl Biotechnol Rep. 2026;13(2):2035-2043. doi:10.30491/jabr.2025.531906.1888

Introduction

The black soldier fly (BSF) larva (*Hermetia illucens*, family Stratiomyidae) is an alternative protein source with promising applications across sectors such as pharmaceuticals, waste management, and animal feed. These larvae are well known for their ability to efficiently convert organic waste into biomass, making them a sustainable and environmentally friendly protein source.[1] BSF larvae contain a high protein content, approximately 35–45% dry weight, making them a valuable raw material for the development of high-value-added products.[2]

BSF cultivation also offers notable advantages in terms of efficiency, requiring significantly less land and resources than conventional livestock farming.[3] In addition, the larvae can grow on various types of household or industrial organic waste, contributing to a circular economy and more sustainable waste management practices. In this context, BSF has been increasingly cultivated for organic waste bioconversion, and in the future, it may become part of a standardized process for waste treatment.[4] Thus, increasing the functional value of BSF larvae through the development of derivative products is essential to support this approach.

One of the methods that can be used to enhance the value of BSF protein is through enzymatic hydrolysis, which produces a complex mixture known as protein hydrolysate. Protein hydrolysates from various biological sources are known to possess a range of bioactivities, including antioxidant, antimicrobial, and tyrosinase inhibitory effects. Tyrosinase is a key enzyme in melanin biosynthesis and is closely associated with pigmentation disorders such as hyperpigmentation.[5] Therefore, compounds that can inhibit tyrosinase are considered promising active agents in skin-lightening cosmetic formulations.

Several synthetic compounds, including hydroquinone and kojic acid, have been used as tyrosinase inhibitors. However, their long-term use has been linked to potential toxicity and adverse effects.[6] This has spurred interest in safer and more natural alternatives. Protein hydrolysates from natural sources, including insects such as BSF larvae, have emerged as attractive candidates. Moreover, hydrolysis methods such as microwave-assisted enzymatic hydrolysis

have been shown to reduce the immunoreactivity of insect proteins and may influence their bioactive potential.[7,8]

Nonetheless, research on the tyrosinase inhibitory activity of BSF protein hydrolysates remains very limited. Previous studies have largely focused on the nutritional profile or antioxidant properties of BSF larvae. Therefore, further research is needed to evaluate the potential of BSF larval protein hydrolysates as natural tyrosinase inhibitors.

Prior studies, for example, Thuraphan et al. (2024), showed that protein and hydrolysates from *Apis mellifera* larvae suppressed collagenase and hyaluronidase, supporting their use in anti-aging cosmetics.[9] Meanwhile, Praseatsook et al. (2022), highlighted that BSF peptide fractions < 3 kDa exerted strong antimutagenic and anti-inflammatory effects.[10] Similarly, Pang et al. (2025), reported that queen bee larvae hydrolysates extended lifespan and enhanced antioxidant defenses in *Drosophila melanogaster*.[11] In addition, Lu et al. (2022), demonstrated that low-molecular-weight peptides from BSF had significantly stronger antioxidant activity compared to larger peptide fractions.[12] In this study, we analyzed BSF larvae across different developmental stages and peptide fractions, based on the premise that protein composition and concentration vary with larval age. Hydrolysis at different stages might produce peptides with distinct characteristics, which may result in differential tyrosinase inhibitory activity.

Based on this background, the present study aims to produce protein hydrolysates from BSF larvae via enzymatic hydrolysis using Alcalase and evaluate their tyrosinase inhibitory activity. The results are expected to contribute to the development of safe, effective, and sustainable natural active compounds for applications in the pharmaceutical and cosmetic industries, while also supporting sustainable waste management practices through BSF cultivation.

Materials and Methods

Materials

The materials, including Black Soldier Fly larvae aged 5, 10, 13, and 15 days old, were purchased from a local BSF farmer in Bandung, West Java, Indonesia. Experimental materials include: n-Hexane, ethanol, methanol, phosphate buffer, Alcalase 2.4 L, L-Tyrosine, Bovine Serum Albumin, O-phthaldialdehyde, mushroom tyrosinase, L-DOPA, Folin-Ciocalteau reagent, and L-Leucine were obtained from Sigma Aldrich, USA.

Preparation and Characterization of BSF Larvae

Black Soldier Fly larvae from each age group were washed, blanched, sorted, and then dried at 50 °C for 24 hours. Subsequently, they were subjected to the defatting process with n-hexane. The process was carried out using the Soxhlet extraction method with n-hexane at a solvent ratio of 1:4 for 6 hours at 70 °C.[13] The resulting oil-solvent mixture from the extraction process was subsequently separated using a rotary evaporator for 2 hours at 60 °C. The percentage of larval oil (w/w) content was determined using the following formula[13]:

$$Oil\ yield = \left(\frac{Weight\ of\ oil\ (g)}{Initial\ sample\ weight\ (g)}\right) x\ 100\%$$

The defatted BSF larvae were then analyzed for total water content following azeotropic distillation and total ash content. For the water content measurement, 200 ml of toluene was saturated with 2 ml of water, and then approximately 5 g of samples were placed into the flask. The flask was heated until the toluene was boiling, and distillation continued for 2 hours. The water volume after distillation was measured, and the water content percentage was determined using the following formula[14]:

$$Water\ content\ = \frac{Water\ volume\ before\ distillation}{Water\ volume\ after\ distillation}\ x\ 100\%$$

The determination of total ash content was done on 2-3 grams of sample using a furnace at 600 ± 25 ℃. The total ash content is calculated based on the weight of the sample used, and the ash content is expressed as a percentage w/w.[14]

$$Total\ ash\ content = \frac{Crucible\ weight\ before\ incandescence\ -\ Crucible\ weight\ after\ incandescence}{Sample\ weight\ (g)}\ x\ 100\%$$

Protein Hydrolysis of Black Soldier Fly Larvae (BSFL)

Enzymatic hydrolysis of protein from defatted BSF larvae was carried out using Alcalase with an activity of 2.4 AU/g. BSF samples from each larval age (10 g) were mixed with distilled water at pH 6.85 (30 ml) in a 1:3 (w/v) ratio at room temperature and hydrated for 2 hours. Enzyme was then added at 2% (w/w) of the larval weight. The enzymatic hydrolysis process was conducted using a rotary evaporator at 60 °C and 100 rpm for 8 hours. Two milliliters of the sample were taken at 1-hour intervals from hour 0 to hour 8. The hydrolysis was terminated by heating the mixture at 90 °C for 15 minutes. The hydrolysate was then centrifuged at 2,500×g for 5 minutes at room temperature, and the supernatant was separated from the precipitate and stored at −20 °C.[15]

Degree of Hydrolysis

The determination of the degree of hydrolysis (DH) was performed using the OPA (o-phthaldialdehyde) method. The OPA reagent was freshly prepared before each analysis. To prepare the OPA reagent, 7.62 g of sodium tetraborate decahydrate and 20 mg of sodium dodecyl sulfate (SDS) were dissolved in 150 ml of deionized water and stirred with a magnetic stirrer until homogeneous. This mixture was then added to 160 mg of OPA powder (97%) previously dissolved

in 4 ml of 95% ethanol. Subsequently, 176 mg of dithiothreitol (DTT, 99%) was added and mixed until homogeneous. The final volume of the OPA reagent was adjusted to 200 ml by adding deionized water. The reagent was stored in a dark bottle at 25 °C.[13]

DH analysis was carried out by mixing 0.4 ml of protein hydrolysate sample (containing 200–300 µg/ml protein) with 3.0 ml of OPA reagent to quantify the amount of free amino acids in the solution. L-Leucine (0.01–0.10 mg/ml) was used as a standard solution to construct the calibration curve. The standard solutions and all samples were incubated at 25 °C for 2 minutes. The absorbance of the standard and sample solutions was then measured at 340 nm using a Shimadzu UV-3101 spectrophotometer. The absorbance values of the samples were converted using the linear regression equation obtained from the standard curve. The degree of hydrolysis was calculated using the following equation[16]:

$$DH = \frac{l1 - l0}{lmax - l0}\ x\ 100\%$$

where DH (%) is calculated relative to L-leucine, l_1 = amino acids present in enzymatic hydrolysate, l_0 = amino acids present in non-hydrolyzed sample and l_{max} = amino acids present in total hydrolysis (acid hydrolysis).

Total hydrolysis of defatted BSF larvae was performed using 6 M HCl. BSF larval samples from each age group (5 g) were mixed with 6 M HCl (15 ml) in a 1:3 (w/v) ratio. The total hydrolysis process was carried out at 100 °C for 24 hours.[16]

Determination of Soluble Protein

The concentration of soluble protein in the hydrolysate obtained from BSF protein hydrolysis was determined using the Bradford method.[17] Bovine Serum Albumin (BSA) was used as a positive control. Standard BSA was dissolved in phosphate-buffered saline (PBS, pH 6.8) and diluted to final concentrations of 30, 60, 90, 120, 150, 180, and 210 µg/ml. A standard calibration curve for BSA was prepared using a 96-well plate, with each well containing 20 µl of the respective BSA concentration diluted in PBS. For the hydrolysate samples, 20 µl was added to each well without additional buffer. In addition to BSA standards and samples, a blank (20 µl PBS and 200 µl Coomassie reagent) was prepared to serve as a reference and to account for baseline absorbance. Each well was then treated with 200 µl of Coomassie Brilliant Blue G-250 reagent, followed by shaking for 30 seconds. The plate was incubated for 3–5 minutes at room temperature before measuring absorbance at 595 nm using a microplate reader. All measurements were performed in triplicate. The absorbance values for the standard concentrations were averaged and used to construct the standard curve, with protein concentration as the x-axis and absorbance as the y-axis. The concentration of soluble protein in the samples was then calculated by inputting the absorbance values into the regression equation obtained from the standard curve.

Tyrosinase Inhibition Assay

The quantitative assay was conducted based on the method described by Masuda et al. (2005) using a 96-well microplate assay. Several reagents were prepared beforehand, including phosphate buffer, tyrosinase enzyme solution, L-DOPA substrate solution, positive control solution (kojic acid), and the test solutions.[18] The percentage of tyrosinase inhibition was determined using a 96-well microplate setting which consists of four different measurements designated as A, B, C, D (Table 1). After incubation, the absorbance was measured using a microplate reader at 475 nm. The percentage of tyrosinase inhibition was then calculated using the following formula[18]:

$$\%Inhibition = \frac{((A - B)\ -\ (C - D))}{A - B}\ X\ 100\%$$

where A is absorbance of blank solution with tyrosinase enzyme, B is absorbance of blank solution without tyrosinase enzyme, C is absorbance of test sample with tyrosinase enzyme and D is absorbance of test sample without tyrosinase enzyme.

Table 1. Determination of Percent Inhibitions

Reagent	Volume per well (µl)			
	A	B	C	D
Phosphate buffer 0.067 M pH 6.8	120	160	80	120
Tyrosinase 300 U/ml	40	-	40	-
Test sample	-	-	40	40
After loading the wells, the plate was incubated for 10 minutes at 25 °C.				
L-DOPA 2.5 mM	40	40	40	40
The plate was incubated again for 10 minutes at 25 °C.				

Fractionation of Larval Hydrolysates Using Solid Phase Extraction (SPE)

The fractionation of BSF larval hydrolysates from D5, D10, D13, and D15 larvae was performed using SPE with a Sep-Pak C18 cartridge (6 cc, 500 mg, Waters). Fifteen milligrams of the sample were dissolved in 1.2 ml of distilled water, followed by filtration using filter paper. The fractionation procedure began with cartridge conditioning using 8 ml of methanol, followed by equilibration with 8 ml of distilled water. After conditioning and equilibration, about 1.2 ml of

the sample solution was loaded into the cartridge. Once the sample had fully passed through the cartridge, a wash step was carried out using 5 ml of distilled water, and the resulting eluate was collected as the aqueous fraction. Subsequent elution was performed using 4 ml of methanol, and the eluate was collected as the methanol fraction. A final wash was then carried out using 8 mL of methanol. All steps from conditioning to washing were repeated three times and assisted with a vacuum pump. The methanol eluates were subsequently concentrated using a rotary evaporator at 50 °C and 100 rpm. Aqueous and methanol fractions were then freeze-dried for 24 hours.

Statistical Analysis

Statistical analysis was performed using ANOVA with a significance level of $p < 0.05$ between the SPE fraction, water fraction, and unfractionated sample.

Results

Characterization of BSF Larvae

The results of characterization can be seen in Table 2. The moisture content of defatted Black Soldier Fly larvae across all age groups was consistently low (0.05%). The total ash content varied only slightly among age groups, ranging from 1.72% to 1.89%, indicating limited mineral or inorganic impurities. Regarding oil yield, substantial variation was observed depending on larval age. The lowest oil content was recorded in D5 larvae (20.22%), whereas the highest was found in D13 larvae (32.22%). This variation is thought to be related to the accumulation of lipids in the later stages of larval development, as the insect transitions toward metamorphosis.

Table 2. Characterization of Defatted BSF Larvae

Parameter	BSFL D5	BSFL D10	BSFL D13	BSFL D15
Total water content (%v/w)	0.05	0.05	0.05	0.05
Oil yield(%w/w)	20.22	24.66	32.22	26.5
Total ash content (%w/w)	1.9	1.72	1.8	1.82

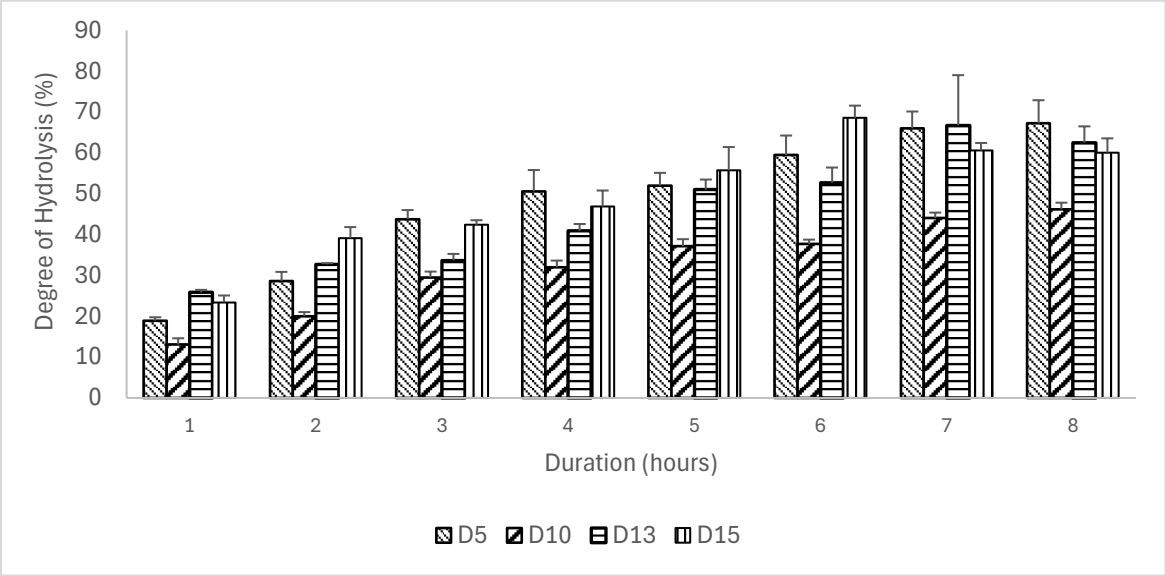

Figure 1. Comparison of the Degree of Hydrolysis (%) Using the OPA Method (n = 3).

Degree of Hydrolysis

The degree of hydrolysis (DH) increased during the 8-hour enzymatic hydrolysis period in all age groups (Figure 1). The highest DH was observed in D5 larvae, reaching 67.24% at the 8th hour. Notably, the D15 samples exhibited a decline in DH after the 6th hour. Early time points (2 hours) already showed DH values between 20.02% and 39.07%, indicating rapid initial cleavage of peptide bonds by Alcalase®. Compared to previous reports of 18.4% DH in a 2-hour hydrolysis of BSF protein, this study achieved higher enzymatic efficiency.[15]

Measurement of Soluble Protein

The initial soluble protein content prior to enzymatic hydrolysis varied significantly across larval age groups (Figure 2). D5 larvae exhibited the highest baseline protein content (12.1253 mg/g), followed by D10 (9.6779 mg/g), D13 (7.6305 mg/g), and D15 (7.4832 mg/g). This trend suggests that younger larvae may possess a higher concentration of soluble proteins, potentially due to their active growth phase and less structural protein development. During the hydrolysis process, soluble protein content

generally increased across all age groups, indicating successful enzymatic breakdown of complex proteins into smaller, soluble peptides. By the 8th hour of hydrolysis, D13 larvae showed the highest increase in soluble protein (13.0568 mg/g), closely followed by D15 (13.0042 mg/g), D5 (10.7884 mg/g), and D10 (10.4832 mg/g). This increase reflects the efficiency of hydrolysis in releasing soluble peptides, which may contribute to bioactivity such as tyrosinase inhibition.

Tyrosinase Inhibition Assay

The tyrosinase inhibition assay revealed a clear positive correlation between hydrolysate concentration and inhibitory activity across all larval age groups (Figure 3). As the concentration increased, so did the percentage of inhibition, indicating a dose-dependent response. At the highest tested concentration (40,000 ppm), the hydrolysate from D15 larvae exhibited the strongest inhibitory effect (88.98%), followed by D10 (82.07%), D13 (80.39%), and D5 (75.77%). This trend suggests that the accumulation of bioactive compounds responsible for tyrosinase inhibition may increase with larval age, potentially due to physiological changes such as protein composition, peptide profile, or secondary metabolite content. The higher inhibition observed in older larvae (D15) could be attributed to the presence of more potent or concentrated inhibitory compounds, possibly linked to their metabolic preparation for metamorphosis. In particular, the methanol eluate from D15 larvae at a concentration of 2,000 ppm achieved up to 17% inhibition, which was notably higher than both the unfractionated hydrolysate and the aqueous fractions. This result implies a possible enrichment of bioactive peptides or phenolic-like compounds in the methanol fraction, which may have been masked or diluted in the crude extract.

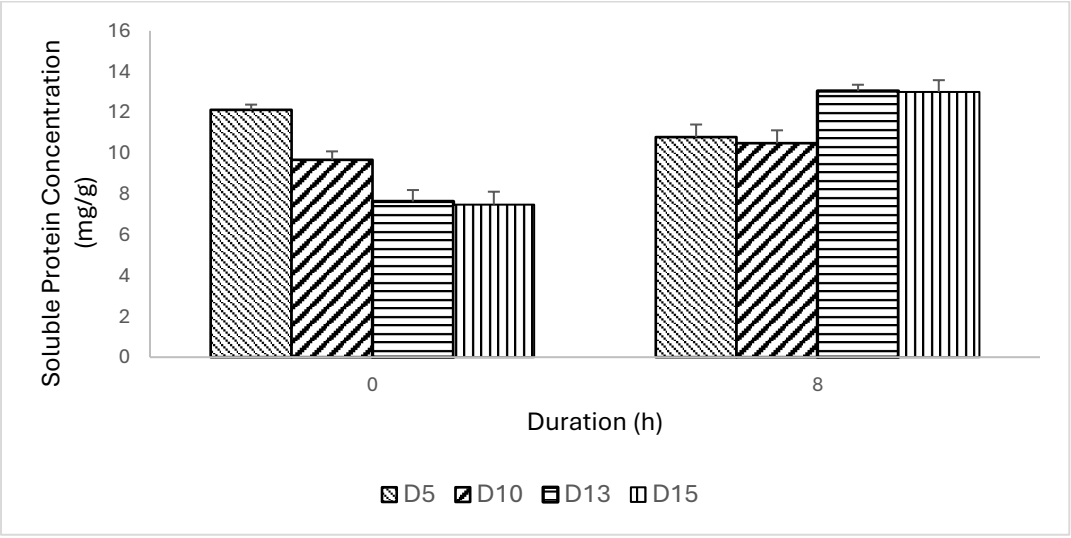

Figure 2. Comparison of Average Soluble Protein Concentration to Hydrolysis Duration, Data Were Obtained from Three Replication (n = 3).

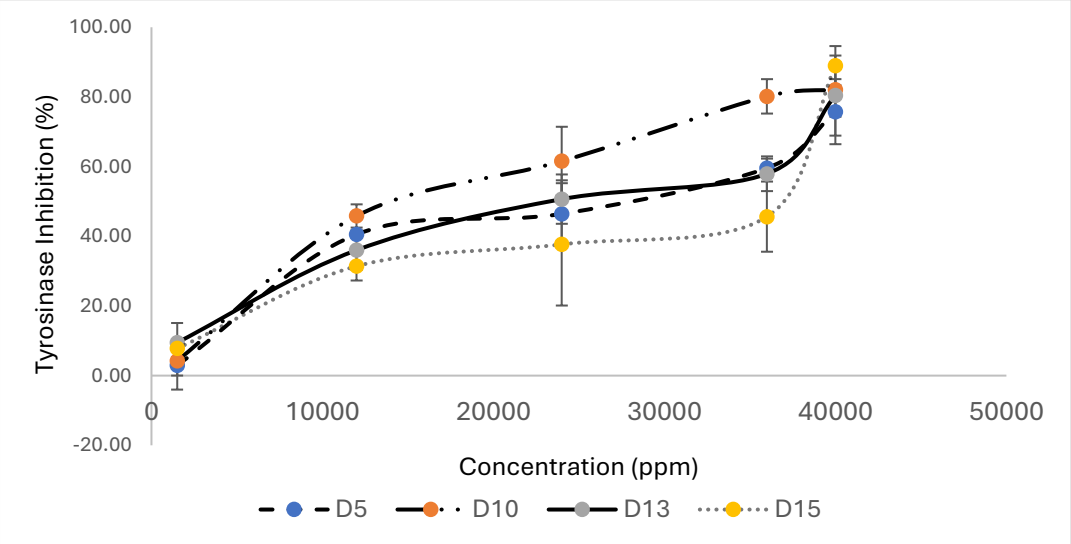

Figure 3. Comparison of the Average Percent Inhibition in Concentration Range from 1,500 to 40,000 ppm (n = 3).

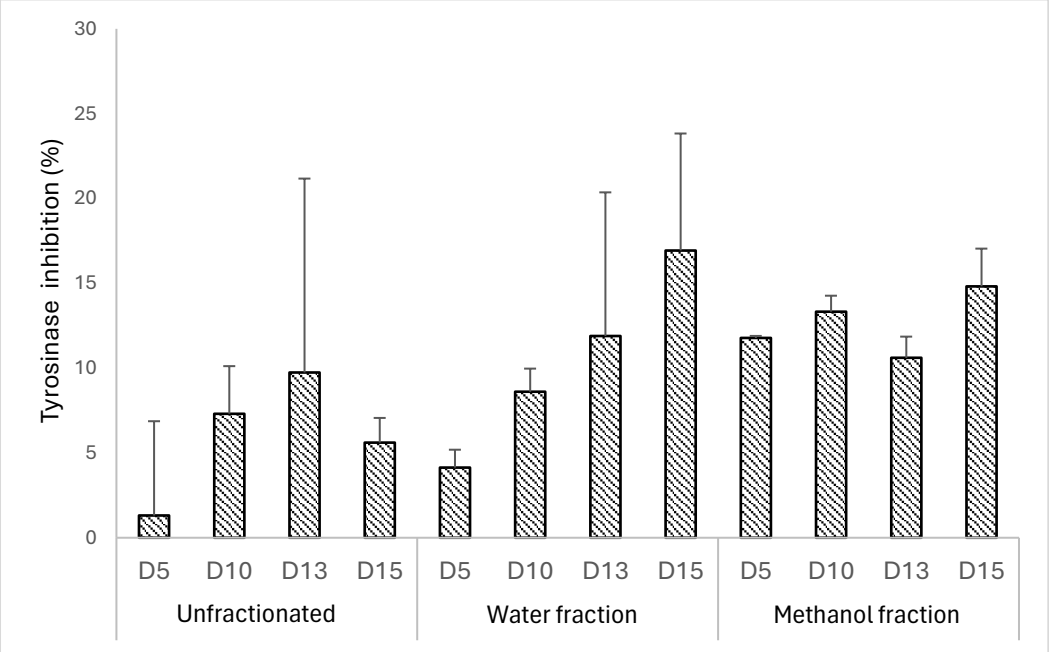

Figure 4. Comparison of Percent Inhibition from Water and Methanol SPE Fraction to Unfractionated Using Same Concentration at 2,000 ppm (n = 3).

Fractionation using Solid Phase Extraction (SPE) enhanced the tyrosinase inhibitory activity of BSF larval hydrolysates. In particular, the methanol fractions consistently showed stronger inhibition and less variation among replicates compared with the aqueous fractions, as reflected by smaller standard deviations (Figure 4). These findings indicate that the inhibitory constituents are more effectively solubilized in methanol. The selection of methanol as the elution solvent was intentional, since its intermediate polarity allows for the recovery of a more specific range of peptides and bioactive compounds with less polarity. Thus, the enhanced inhibition observed in methanol fractions can be attributed to the less polar peptides. The enhanced activity observed in the methanol fraction aligns with previous findings that certain hydrophobic or semi-polar compounds, including peptides with aromatic residues, can contribute to tyrosinase inhibition.[19] Moreover, the relatively uniform activity across replicates in the methanol fraction suggests a more stable and concentrated presence of these inhibitory compounds post-fractionation.

Discussion

The physicochemical characterization confirmed that BSF larvae provide a stable raw material due to their low moisture and ash content, ensuring minimal degradation and contamination risk during processing. The observed variation in oil content between age groups highlights the importance of harvest timing to optimize lipid or protein yields, as older larvae accumulate higher fat reserves essential for metamorphosis. The oil content in BSF larvae varied depending on the larval age, with the lowest oil content observed at D5 larvae (20.22%) and the highest at D13 larvae (32.22%). The variation in oil content among BSF larvae was also reported by Muangrat & Pannasai (2024), who obtained an oil content of 25.99% using Soxhlet extraction, and by Firmansyah & Abduh (2019), who reported an oil content of 35.5%.[13,20] However, the exact age of larvae used in these previous studies was not specified. The defatting process increases the crude protein content and reduces fat content in the larvae, which may lead to partial or total degradation of amino acids such as cysteine, tryptophan, and methionine.[21]

The degree of hydrolysis (DH) is one of the key parameters in the hydrolysis process, representing the percentage of cleaved peptide bonds. The increasing degree of hydrolysis over time indicates that Alcalase® effectively breaks peptide bonds in BSF proteins. The rapid rise in DH within the first 2 hours suggests that accessible cleavage sites are abundant in the early hydrolysis phase. The later decline in DH in D15 larvae may be attributed to structural changes in proteins or substrate depletion, limiting further hydrolysis.

Enzymatic hydrolysis is conducted to enhance the bioactivity of the hydrolysate by converting proteins into peptides while retaining their nutritional value.[22] In this study, the DH of BSF larvae across different ages tended to increase from hour 0 to hour 8, although a decrease was observed after hour 6 in D15 larvae. The highest DH was recorded in D5 larvae at hour 8, reaching 67%, as shown in Figure 1. This result is slightly higher than that reported by Batish et al. (2020), who performed hydrolysis using Alcalase for 2 hours and obtained a DH of 18.4%, whereas the DH range at hour 2 in this study was between 20.02% and 39.07%.[15] A longer hydrolysis duration increases the contact time between enzyme and substrate, leading to a

higher number of hydrolyzed peptide bonds. Additionally, both the duration and intensity of hydrolysis contribute to an increase in DH.[23]

Determination of soluble protein in this study is using the Bradford Method, which is based on a shift in the absorbance of Coomassie Brilliant Blue G-250 dye. In the Bradford method, the changes color from red to blue happen under acidic conditions when binding to proteins, then measured at a wavelength of 595 nm.[24] The soluble protein content trends further emphasize the role of larval development stage. D13 larvae displayed the greatest increase in soluble protein during hydrolysis, indicating higher hydrolysis efficiency and potentially higher release of bioactive peptides. The fluctuations in D15 larvae suggest incomplete or variable enzyme access to proteins during prolonged incubation.

The protein content of BSF larvae can vary depending on the larval age. In a study by Rachmawati et al. (2010), D5 larvae contained approximately 61.42% crude protein, D10 larvae contained around 44.44%, and D15 larvae around 44.01%.[25] In this study, the higher soluble protein content observed in D5 larvae compared to the D10 larvae may be related to the early growth phase of the larvae. The decrease in protein content in D15 larvae is likely associated with metabolic changes as the larvae prepare to enter the prepupal stage.

In young larvae, physical growth occurs rapidly; once growth plateaus, only lipid composition tends to change. The high protein content in younger larvae may also be attributed to structural proteins such as those in the cell wall. The quality and quantity of BSF larval protein are influenced by the insects developmental stage, which also affects the larval lipid composition. High lipid content during the prepupal stage may result from metabolic changes during metamorphosis. In addition to age, the type of feed provided to BSF larvae during development also influences nutrient content.[26] In a study by Pamintuan et al. (2019), larvae fed with vegetable waste contained about 31.95% protein, whereas larvae fed with snakehead fish offal had around 38.95%.[27]

Overall, the soluble protein content in BSF larvae fluctuated across different ages throughout the hydrolysis process, though it generally increased over time up to the 6th hour. As shown in Figure 2, at the beginning of hydrolysis (hour 0), the highest protein content was found in D10 larvae (12.1253 mg/g), while the lowest was in D13 larvae (7.6305 mg/g). As hydrolysis progressed, the protein content in D13 larvae increased significantly, becoming the highest by the 8th hour (13.0568 mg/g), indicating high hydrolysis efficiency at this age. A similar trend of increasing soluble protein content with prolonged hydrolysis duration was also observed by Palupi et al. (2011), who found that longer hydrolysis times promoted the release of peptide bonds and increased protein solubility.[23]

In contrast, D15 larvae showed fluctuating soluble protein levels throughout the hydrolysis period, possibly due to incomplete peptide bond cleavage during the process. Meanwhile, D5 and D10 larvae showed relatively stable protein levels from the start to the end of hydrolysis. These findings suggest that larval age affects the enzyme's effectiveness in releasing soluble proteins, with D13 and D15 larvae showing the most responsive hydrolysis behavior, particularly after prolonged incubation.

The tyrosinase inhibition results demonstrated a clear dependence on both hydrolysate concentration and larval age. Inhibitory activity of the larval protein hydrolysates against tyrosinase increased with rising concentrations across all larval ages (D5, D10, D13, and D15). The stronger inhibition observed in D15 samples suggests that advanced larval development yields peptides or phenolic compounds with higher affinity to the tyrosinase active site. Notably, the inhibition profiles differed among larval ages, particularly at intermediate concentrations (10,000–25,000 ppm), where D10 and D13 showed relatively higher inhibitory effects compared to D5 and D15 (Figure 3). This variation may be attributed to differences in protein composition, peptide size distribution, or specific inhibitory sequences generated during enzymatic hydrolysis at different larval developmental stages. Overall, the results indicate that both the larval age and the concentration of the hydrolysate significantly influence tyrosinase inhibition.

Besides, the enhanced inhibitory activity in the methanol SPE fractions supports the hypothesis that fractionation selectively enriches active compounds such as hydrophobic peptides and low molecular weight phenolics, improving inhibitory potency. Statistical analysis using ANOVA with $p < 0.05$ between methanol fraction, water fraction and unfractionated shows that there is a significant difference between methanol fraction from D10 larvae against water fraction and unfractionated. Meanwhile, methanol and water fraction from D15 larvae show significant difference compared to unfractionated samples. Overall, these findings underscore the potential of BSF larval hydrolysates as a natural source of tyrosinase inhibitors, supporting further development for pharmaceutical and cosmetic applications.

Although soluble protein content increased during enzymatic hydrolysis across all larval age groups, tyrosinase inhibition did not always correlate with the amount of soluble protein. For example, D5 larvae showed the highest initial soluble protein content, yet inhibitory activity of its hydrolysate was lower than that of older larvae such as D13 and D15. Hydrolysates from D15 larvae exhibited higher specific activity than earlier stages; however, their lower soluble protein content presents a yield limitation. To address this, process improvements such as optimizing hydrolysis conditions or employing scalable purification

techniques (e.g., ultrafiltration or adsorption resins) may help compensate for reduced yield. Thus, while the lower yield poses a challenge, the strong bioactivity profile of D15 hydrolysates supports their potential for further development.

The lack of correlation between soluble protein content and tyrosinase inhibition indicates that overall protein yield is not a reliable predictor of bioactivity. Bulk protein assays such as Bradford provide quantitative estimates but do not capture structural or functional attributes that determine inhibitory potency. It is plausible that only a limited subset of low-molecular-weight, moderately hydrophobic peptides accounts for most of the observed activity, while larger or inactive proteins inflate protein measurements without contributing to inhibition. This interpretation aligns with the enhanced activity consistently observed in the methanol fractions, which are more likely to enrich peptides of intermediate polarity capable of interacting with tyrosinase.[19]

Additional contributors, such as protease-resistant peptides or co-eluting non-peptidic metabolites, may further obscure any linear relationship between protein levels and inhibitory effect. These findings suggest that enzyme inhibition is governed primarily by the specific nature of the bioactive molecules recovered during fractionation, rather than by total protein yield, showing the need for activity-guided isolation and molecular characterization.

An important point to consider when interpreting this inverse trend is that the total amount of soluble protein may be less relevant than the types of peptides being generated. As larvae develops, proteins are progressively digested into smaller fragments, and certain short peptides, particularly those enriched in hydrophobic or aromatic residues, can interfere with tyrosinase activity more effectively than bulk protein.[9] At the same time, older larvae, especially at the prepupal stage, may accumulate additional bioactive molecules such as phenolics or metabolites associated with cuticle formation and melanization, which could co-elute with protein fractions and contribute to the observed inhibition. This interpretation aligns well with larval developmental biology. During the transition toward prepupa, major metabolic shifts occur, including changes in amino acid and fatty acid pools as well as increased deposition of secondary metabolites.[28] Hsiao et al. (2022) reported stage-specific differences in the metabolite profile of *Hermetia illucens* larvae, with compounds such as phenylalanine, arginine, and oleic acid varying across instars, potentially explaining the stronger activity observed in later stages.[29] Midgut physiology studies further demonstrate that enzyme activity and gut pH change during larval development, which could affect the generation and stability of these bioactive peptides.[30] Supporting this view, recent work fractionating BSF larval hydrolysates by molecular weight showed that peptides < 3 kDa displayed strong antimutagenic and anti-inflammatory activity, whereas larger fractions were more associated with antioxidant effects.[10] Taken together, these findings suggest that both peptide size distribution and the accumulation of non-protein metabolites in older larvae may underline the enhanced tyrosinase inhibition despite lower total protein levels.

Conclusion

The findings of this study indicate that the tyrosinase inhibitory activity of *Hermetia illucens* (BSF) larval protein hydrolysates is influenced by both larval age and the application of fractionation. Among all samples tested at 40,000 ppm, the highest tyrosinase inhibition was observed in the D15 hydrolysate (88.98%), followed by D13 (87.78%), D10 (82.07%), and D5 (75.77%). Furthermore, at a lower concentration (2,000 ppm), hydrolysates that underwent solid-phase extraction (SPE) fractionation exhibited greater tyrosinase inhibitory activity compared to unfractionated samples, suggesting that fractionation enhances the bioactivity of the hydrolysates.

Authors' Contributions

HP: Conceptualization, methodology, original draft preparation, manuscript review and editing, funding acquisition, supervision; FST: Performed research, data acquisition, analyzed data, original draft preparation, manuscript review, and editing; DR: Methodology, supervision, manuscript review, and editing. All the authors read and approved the final version of manuscript for submission.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

This study was financially supported by Directorate General of Higher Education, Ministry of Education, Culture, Research, and Technology, Republic of Indonesia under contract no. 337/IT1.B07.1/SPP-LPPM/VI/2023.

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