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Antioxidant Activity of Protein Hydrolysate from Mole Crab (*Emerita* Sp.): In Vitro and In Silico Studies

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A B S T R A C T

This study aims to produce antioxidant potential from Emerita sp. protein hydrolysate through in vitro and in silico approaches. The hydrolysis process was carried out using alcalase enzymes. Antioxidant activity was evaluated by DPPH and ABTS methods. The results showed that at a concentration of 250 µg/mL, the free radical inhibition activity of DPPH reached 85.67%, and ABTS was 78.54%. The in silico approach showed that the main bioactive peptides such as Gly-His and Pro-Leu have high affinity for free radical-producing enzymes. These peptides have the potential to be developed as basic ingredients for antioxidant nutraceuticals.

Keywords: antioxidant, emerita sp., protein hydrolysate, in vitro, in silico

1. Introduction

Indonesia's marine resources hold enormous biological potential to be utilized as raw materials for functional food products, including natural antioxidants. One marine organism that has not been widely studied but has potential is mole crab (*Emerita* sp.). This organism is known to have a high protein content that has the potential to be hydrolyzed into bioactive peptides with various physiological activities, including as an antioxidant (Kim and Wijesekara, 2010).

Antioxidants are compounds that can neutralize free radicals, which are unstable molecules that can cause cell and tissue damage through oxidative stress. Oxidative stress has been linked to various degenerative diseases such as cancer, diabetes, and cardiovascular disease (Lobo *et al*., 2010). Free radicals are reactive molecules that have unpaired electrons. The presence of free radicals in excessive amounts can cause oxidative stress, which damages cellular structures and is the main cause of various degenerative diseases such as cancer, cardiovascular disease, and premature aging. To overcome this, the body requires antioxidants, both endogenous and exogenous, to neutralize the damaging effects of free radicals.

Synthetic antioxidant sources such as BHT and BHA are often used in the food and pharmaceutical industries, but long-term use of these chemicals can cause toxic effects. Therefore, the search for safe, effective, and environmentally friendly natural antioxidants is a major focus in the development of nutraceutical products today. Therefore, exploration of natural antioxidant sources from the sea is important as an alternative to synthetic antioxidants that have long-term side effects (Frijhoff *et al*., 2015).

Enzymatic hydrolysis of proteins can produce bioactive peptides that have antioxidant activity, depending on the amino acid composition, size, and secondary structure of the peptide. Several studies have shown that peptides containing hydrophobic amino acid residues or those rich in histidine, tyrosine, and methionine show high antioxidant activity (Sarmadi and Ismail, 2010). Protein hydrolysis with enzymes such as alcalase can break down proteins into small peptides that are more easily absorbed and have the potential to act as antioxidants. In addition to the laboratory test approach (in vitro) and in silico methods such as molecular docking can be used to estimate the potential interaction between peptides and target enzymes. This study aims to produce protein hydrolysates from *Emerita* sp. and examine their antioxidant activity using in vitro methods (DPPH and ABTS) and in silico approaches.

2. Materials and Methods

2.1 Materials

The materials used in this study were the mole crab (*Emerita* sp.) on the South Coast of Java Island, alcalase enzyme, distilled water, HCl (Merck) 0.1 N, NaOH 0.5 N, phosphate buffer solution 0.2 M pH 8, DPPH reagen, ABTS reagen, and potassium persulfate. The tools used in this study were a blender (Philips HR 1364), oven (Memmert), centrifuge (Himac CR 21G), waterbath (Memmert), 500 mL beaker glass, pH meter, Fourier Transform Infrared

Spectroscopy, UV-Vis Spectrophotometer (Thermo Spectronic), LC-MS (Liquid Chromatography Mass Spectrometry) (Thermo Spectronic), software BIOPEP-UWM, Peptide Ranker, and Auto Dock (HDOCK).

2.2 Protein Hydrolyzate Production

The manufacture of protein hydrolyzate using the enzymatic hydrolysis method refers to Shaibani *et al*. (2020), modified using mole crab (*Emerita* sp). The first step in making protein hydrolyzate is mole crab (*Emerita* sp.) was washed thoroughly, drained, and then chopped using a grinder. The sample was mixed with deionized water (1:2, w/v), homogenized, and heated at 85°C for 20 minutes to inactivate endogenous enzymes. The solution was then adjusted to a basic pH by adding phosphate buffer solution 0.2 M pH 8. The hydrolysis process was carried out using alcalase enzyme with concentrations of 1%, 2%, and 3% of the sample weight, at a temperature of 50°C for 2 hours. The hydrolysate was then heated at 95°C for 15 minutes to inactivate the enzyme, then centrifuged at 8000 rpm for 10 minutes to obtain the supernatant as a protein hydrolysate. The hydrolysate is then placed in a refrigerator to prevent damage and degradation.

2.3 Antioxidant Analysis Using DPPH (Shah and Modi, 2015)

The antioxidant activity assay using the DPPH method was conducted based on the study by Shah and Modi (2015) with slight modifications. The assay began with the preparation of DPPH reagent by dissolving 0.002 grams of DPPH powder in 57 mL of methanol. The absorbance of the reagent was measured at a wavelength of 517 nm using a spectrophotometer until it reached a value between 1.0 and 1.2. Sample solutions were prepared at concentrations ranging from 50 to 250 µg/mL. Each sample (0.5 mL) was mixed with 4.5 mL of DPPH reagent in a test tube. The blank solution was prepared by mixing 0.5 mL of methanol with 4.5 mL of DPPH reagent. Both solutions were vortexed and incubated for 60 minutes in the dark at room temperature. After incubation, the absorbance of the samples and the blank was measured again at 517 nm using a spectrophotometer. The calculation of antioxidant levels for the DPPH method is expressed as % Antioxidant which is calculated using the following formula:

Antioxidant (%) = (blank absorbance value – sample absorbance value)/(blank absorbance value) x 100% (1)

2.4 Antioxidant Analysis Using ABTS (Islam et al., 2022)

The antioxidant activity assay using the ABTS method was carried out based on the procedure described by Islam *et al*. (2022), with some modifications. The assay began by dissolving 0.003 grams of ABTS powder in 5 mL of distilled water and mixing it with 0.003 grams of potassium persulfate dissolved in another 5 mL of distilled water to produce the ABTS stock solution. The working ABTS reagent was then prepared by diluting 0.4 mL of the stock solution with 12.6 mL of methanol. The absorbance of the reagent was measured at 734 nm using a spectrophotometer until it reached approximately 0.78. Sample solutions were prepared at concentrations ranging from 50 to 250 µg/mL. Each sample (0.5 mL) was mixed with 4.5 mL of ABTS reagent in a test tube. A blank solution was prepared by mixing 0.5 mL of methanol with 4.5 mL of ABTS reagent. Both sample and blank solutions were homogenized using a vortex mixer and incubated for 1 minute. The absorbance of each solution was then measured at 734 nm using a spectrophotometer. The calculation of antioxidant levels for the ABTS method is expressed as % Antioxidant which is calculated using the following formula:

Antioxidant (%) = (blank absorbance value – sample absorbance value)/(blank absorbance value) x 100% (2)

2.5 Fourier-Transform Infrared Spectroscopy (FTIR) Analysis (Kristoffersen et al., 2019)

A 7.5 µL protein hydrolysate sample was placed and deposited on a plate (96-well IR transparent Si), then dried at room temperature for 30 min to form a dry film layer. Five small portions of each protein hydrolysate sample were taken to make repeat measurements. FTIR assay was performed using High Throughput Screening eXTension (HTS-XT) combined with a Tensor 27 spectrometer. The measured spectra were in the range of 4000 and 400 cm-1 using a spectral resolution of 4 cm-1 and an aperture of 5.0 mm.

2.6 In Silico Analysis

Protein sequences obtained from the LC-MS/MS peptide data were analyzed to identify bioactive peptides using the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/en/biopep-uwm-2/). This analysis was carried out according to Minkiewicz et al. (2019). Bioactivity, protein sequences, number and location of peptides were obtained from protein sequences identified by the "profiles of potential bioactivity" menu in the BIOPEP-UWM database. Then to determine the peptide activity ranking using peptide ranker (http://distilldeep.ucd.ie/PeptideRanker/). Peptide ranker is a server that is able to rank peptides according to the level of peptide activity. Peptide ranker scores are based on the prediction of peptide bioactivity. Peptide ranker can predict the probability (between 0 and 1) of a peptide being bioactive. The 3D structures of amino acids identified from biopeptide analysis were constructed using UCSF Chimera, energy-minimized, and saved in PDB format. The target protein structure was downloaded from the RCSB PDB database (https://www.rcsb.org/), refined by removing non-standard ligands and adding hydrogen atoms. Molecular docking was performed using the HDOCK server (http://hdock.phys.hust.edu.cn/) by uploading the prepared ligand and protein files. The resulting binding affinity and RMSD values were sent via email and further analyzed using Discovery Studio to visualize molecular interactions.

3. Results and Discussions

3.1 In Vitro Antioxidant Activity

Antioxidant activity test using the DPPH method involves the compound 1,1-diphenyl-2-picrylhydrazil which is an artificial compound with a role as a free radical in the antioxidant test aimed at determining the ability of protein hydrolysates to ward off oxidants or free radicals. The ABTS antioxidant activity test utilizes the compound 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) which is added to the sample and acts as an oxidant that will be suppressed by the sample compound suspected of having antioxidant properties.

The value of antioxidant activity using the DPPH and ABTS method on mole crab protein hydrolysates (*Emerita* sp.) can be seen in Table 1.

Table 1. Antioxidant Activity of Mole Crab Protein Hydrolysate (DPPH)

Concentration (µg/mL)	DPPH (%)	ABTS (%)
50	35.2	28.3
100	48.7	43.9
150	62.1	59.8
200	74.6	69.1
250	85.67	78.54

Information:

1. Data is the mean of 3 replications ± standard deviation
2. Data followed by different lowercase letters showed a significant difference (p<5%)

The results of in vitro antioxidant activity tests showed that protein hydrolysates from mole crab (*Emerita* sp.) had the ability to capture free radicals which increased significantly with increasing concentration. Based on Table 1, DPPH radical activity increased from 35.2% at a concentration of 50 µg/mL to 85.67% at 250 µg/mL, while ABTS activity increased from 28.3% to 78.54% at the same concentration. This increasing pattern indicates a positive dose-response relationship, indicating the effectiveness of bioactive compounds in the hydrolysate. In addition, the IC50 value for the DPPH method of 85.67 µg/mL is included in the strong category, in accordance with the classification of antioxidant activity which states that IC50 values below 100 µg/mL reflect high antioxidant potential. This shows a positive relationship between hydrolysate concentration and antioxidant effectiveness, indicating that the active compounds in the hydrolysate work in a dose-responsive manner in capturing free radicals. According to Saefudin *et al*. (2013) the percentage of free radical inhibition is divided into several classifications. Antioxidant inhibition with a value of > 90% is included in high activity, 50% - 90% is included in high, 20% - 50% is included in medium, and < 20% is included in low. Abstain inhibition values such as 0% indicate that a compound does not show antioxidant activity.

3.2 In Silico Analysis

The results of the in silico analysis of the mole crab protein hydrolyzate with the addition of alcalase enzime can be seen in Table 2.

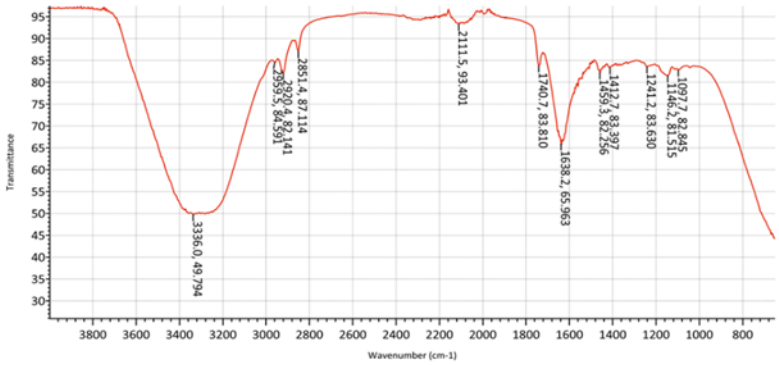
Table 2. Results of In Silico Analysis of Mole Crab Protein Hydrolyzate

Peptide	Enzyme Target	Bond Energy (kcal/mol)
Gly-His (GH)	NADPH oxidase	-7.89
Pro-Leu (PL)	Myeloperoxidase	-8.32

In silico analysis showed that two main peptides identified from *Emerita* sp. protein hydrolysate, namely Gly-His (GH) and Pro-Leu (PL), have potential as antioxidant agents based on their binding affinity to target enzymes involved in oxidative stress. Molecular docking results showed that GH has a binding energy of -7.89 kcal/mol to NADPH oxidase, while PL shows a lower binding energy, namely -8.32 kcal/mol to Myeloperoxidase. The negative and fairly low binding energy value indicates a stable interaction between the peptide and the target enzyme, which has the potential to inhibit the activity of the free radical-producing enzyme. NADPH oxidase and Myeloperoxidase are two main enzymes that play a role in the formation of reactive oxygen species (ROS), so inhibition of these enzymes can reduce the level of oxidative stress in the body (Aratani, 2018).. The strong binding affinity of these two peptides indicates that GH and PL have the potential to be natural inhibitors of these enzymes, supporting previous in vitro findings showing high antioxidant activity of *Emerita* sp. hydrolysate.

3.3 FTIR Results of Mole Crab (Emerita sp.) Protein Hydrolysate

The results of the FTIR of mole crab protein hydrolyzate with the addition of alcalase enzime can be seen in Picture 1. and Table 3.



Picture 1. Results of FTIR of Mole Crab Protein Hydrolyzate

Table 3. Results of FTIR of Mole Crab Protein Hydrolyzate

Peak Number	Wave Number	Intensity
1	1097.7	82.845
2	1146.2	81.515
3	1241.2	83.630
4	1412.7	83.397
5	1459.3	82.256
6	1638.2	65.963
7	1740.7	83.810
8	2111.5	93.401
9	2851.4	87.114
10	2920.4	82.141
11	2959.5	84.591
12	3336.0	49.794

The FTIR test results of the protein hydrolysate of sea antlers with the addition of alcalase enzyme showed the presence of main functional groups such as hydroxyl (-OH), amino (-NH), carbonyl (C=O), carboxylate ($-COO^-$), and methyl (CH_3) groups. The intense peak at 1638.2 cm^{-1} (Amide I) indicates an effective protein hydrolysis process, producing short peptides and amino acids. The peaks at 2959.5 cm^{-1} and 2851.4 cm^{-1} indicate the presence of aliphatic groups from amino acids, while other peaks such as 1241.2 cm^{-1} and 1146.2 cm^{-1} indicate the possibility of carbohydrate residues or other polar components. The addition of alcalase enzyme provides good hydrolysis results because it changes the protein structure into a simpler form that is more accessible for functional or health food applications.

4. Conclusions

The conclusions that can be drawn from the results of the analysis of the in vitro an in silico of mole crab protein hydrolyzate using alcalase enzime are protein hydrolysate from *Emerita* sp. showed significant antioxidant activity both in vitro and in silico. In vitro test showed an increase in DPPH and ABTS radical scavenging activity as the concentration increased, with an IC50 value of 85.67 µg/mL which is quite strong. Meanwhile, in silico analysis successfully identified Gly-His and Pro-Leu peptides as the main candidates with high antioxidant activity potential based on the results of modeling and prediction of molecular interactions. These findings indicate that protein hydrolysate from Emerita sp. has potential applications as active ingredients in nutraceutical products and health supplements, and can be the basis for the development of environmentally friendly and sustainable natural peptide-based therapies.

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