In-Silico Approach of Mole Crab (*Emerita* sp.) Peptides Produced by Alcalase Hydrolysis

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ABSTRACT

One type of mole crab in Indonesia is *Emerita* sp., which has a fatty acid content of 3.57% and crude protein content of 32.42% (100 mg). The use of mole crabs is currently limited to food sources; therefore, it is necessary to conduct research to optimize the use of mole crabs, which are a source of protein hydrolysate. The samples were used under fresh conditions and stored at -20°C before processing. This study aimed to produce protein hydrolysates from moles of crabs. This hydrolysate is produced by enzymatic hydrolysis of marine back-down raw materials using alcalase. In silico analyses have identified the potential of marine-receding protein hydrolysates. The results of in silico analysis using BIOPEP and Peptide Ranker revealed that these peptides exhibited multiple bioactivities, including ACE inhibition, DPP-IV inhibition, and antioxidative and anti-inflammatory effects. The dipeptide PW (Pro-Trp) achieved the highest Peptide Ranker score of 0.993, with a predicted dual function as an antioxidant and DPP-IV inhibitor. Molecular docking confirmed strong binding affinities to target receptors, with the AF peptide displaying the best interaction against ACE (-129.70 kcal/mol) and GH peptide against DPP-IV (-113.68 kcal/mol). These results suggest that mole crab hydrolysate contains promising peptides with potential applications as nutraceuticals, particularly in the management of hypertension and type 2 diabetes mellitus. The highest potency based on the in-silico peptide hydrolysate has a strong antihypertensive effect. Further in vivo research is needed to explain the potential of mole crab peptides as bioactive antihypertensive agents in peptide form.

Keywords: bioactive peptides, enzymatic hydrolysis, nutraceuticals, computational prediction

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Introduction

The fisheries and marine sectors in Indonesia have great potential to be developed as sources for meeting the nutritional needs of the community. One of the fishery commodities with a fairly high nutritional content is crustacean species such as mole crabs, which belong to the *Hippidae* family and live in the intertidal zone (swash zone). Mole crabs are widely found in Indonesia, including *Emerita* sp. The use of mole crabs as a food ingredient has long been carried out by the community around the southern coast of Central Java, and it has become a source of income for several fishermen in the area, either for family consumption or sold as snacks and sea bream because it has a fairly high nutritional content, especially protein and omega-6 content (Santoso *et al*., 2015).

Mole crabs are one of the largest communities living on sandy beaches from spring to autumn. Mole crabs (*Emerita* sp.) inhabit sandy coastal areas year-round, but their population tends to be more abundant during certain periods, depending on local environmental conditions such as tides, temperature, and plankton availability (Wardiatno *et al.*, 2015; Dugan & Hubbard, 2010).In terms of the diversity of mole crab in Indonesia, there are seven superfamily species that have been discovered, namely *Emerita emeritus*, *Hippa adactyla*, H. *admirabilis*, H. *marmorata*, H. *ovalis*, H. *kelaino*, and *Albunea symmista*. In the discovery, it was explained that only three species were found on the island of Java, namely, E. *emeritus*, H. *adactyla*, and A. *symmista*. The percentage of populations of these three species on Java was 70.5-75.3% for E. *emeritus*; 22.5-24.7% for H. *adactyla*, and 2.2-4.8% for A. *symmysta*. However,

the most common and widespread species worldwide is *Emerita analoga* (Wardiatno *et al*., 2015).

Mole crab (*Emerita* sp.) has been shown to have a high nutritional content with a fat content of 17.22-21.56%, omega-3 (EPA and DHA) of 7.75-14.48%, omega-6 of 12.94%, linoleic acid of 11.11%, and arachidonic acid of 1.83%, which is quite high compared to several other types of crustaceans (e.g., shrimp, lobster, and crab). The EPA content of mole crab is 6.41-8.43%, which is higher than the DHA content of 1.34 - 6.57% (Haq *et al*., 2018). Mole crabs can also be utilized as food to reduce cholesterol in the body by reducing 47-53% in BALB/C. In addition, the chemical contents of raw mole crabs include 9.1% water, 32.5% crude protein, 10.2% fat, 4.9% crude fiber, 26.4% ash, 9.3% calcium, and 1.6% phosphorus on a dry basis (Haq *et al*., 2018). Recent studies have shown that the mole crab content is dominated by a protein content of 35-38% and ash content of 31-35% (Santoso *et al*., 2015). The results were not significantly different from those of other crabs in general, namely protein content of 34-37% and ash content of 24-38%. The high ash content in mole crabs is thought to be due to the composition of the shell, which covers 50% of the body (Haq *et al.* 2018). The high nutritional content makes mole crab one of the foods that has the potential to be used as a source of nutrition, especially protein. The use of traditional processed foods, such as "rempeyek" or fried "yutuk", needs to be innovated and deepened to make better use of mole crabs, one of which is to make protein hydrolysate from mole crabs. Mole crabs, belonging to the genus *Emerita*, are marine crustaceans that have recently attracted attention as underutilized seafood resources that are rich in proteins and bioactive compounds. Research in the past five years has emphasized the growing interest in valorizing marine by-products and less-exploited species, such as mole crabs, to produce high-value functional ingredients, such as protein hydrolysates (Sun *et al*., 2023; Li *et al*., 2021).

Protein hydrolysates are produced via enzymatic hydrolysis, which breaks down proteins into smaller peptides and free amino acids. This process improves digestibility and bioavailability, while generating bioactive peptides with antioxidant, antimicrobial, antihypertensive, and immunomodulatory properties (Wang *et al*., 2022). Studies have indicated that proteins from crustaceans, including mole crabs, contain unique peptide sequences that can provide significant health benefits beyond basic nutrition (Aleman *et al*., 2020). These studies indicate its potential as a novel source for protein hydrolysate development, one of which is to produce protein hydrolysate from mole crabs.

Protein hydrolysate is the result of the breakdown of proteins into simple peptides and amino acids through hydrolysis. Protein hydrolysis can be performed chemically or enzymatically. The advantages of enzymatic protein hydrolysis over chemical methods have been well documented in scientific literature. Enzymatic hydrolysis is preferred because of its mild operating conditions, specificity, and ability to preserve the essential amino acids. In contrast, chemical hydrolysis, particularly under harsh conditions, can lead to degradation of certain amino acids (Martínez-Medina *et al*., 2022; Sun *et al*., 2023). Protein hydrolysis is affected by the concentration of hydrolyzing materials (enzymes), temperature, pH, and time (Hunsakul *et al*., 2022, 2024; Sajib *et al*., 2020). Protein hydrolysate can be used as a flavor enhancer and as a source of protein and amino acids in food (Li *et al*., 2021; Wang *et al*., 2022; Zhuang *et al*., 2023. Protein hydrolysates have been used as nutraceuticals to treat various diseases, including cancer, cardiovascular diseases, and inflammatory conditions. Many review articles have reported that marine organisms (including crab (*Portunus pelagicus*) and shrimp (*Litopenaeus vannamei*)) and vegetable protein hydrolysates exhibit multiple bioactivities such as antioxidant, antidiabetic, antihypertensive, antimicrobial, and opioid agonist activities, which are primarily attributed to the enhancement of radical scavenging and enzyme inhibitory actions (Shaibani *et al*., 2020; Gao *et al*., 2020). These peptides have been shown to exhibit antioxidant, antimicrobial, and immunomodulatory activities, making them valuable functional ingredients in food and nutraceutical applications (Li *et al*., 2021; Wang *et al*., 2022). The enzymatic hydrolysis of mole crab proteins not only improves digestibility and nutritional value but also generates bioactive compounds that may support health beyond basic nutrition. Thus, the utilization of mole crab protein hydrolysates can provide both nutritional and therapeutic benefits, highlighting their importance in food science and health industries (Sun *et al*., 2023). Therefore, the use of mole crabs in protein hydrolysates is very important because it adds benefits in addition to being a source of nutrition, namely, used for health. Protein hydrolysates are generated through the enzymatic cleavage of proteins, resulting in peptides with potential bioactivity, such as antioxidant or antihypertensive effects (Gao et al., 2021). To obtain the characteristics and potential of mole crab protein hydrolysate peptides as a source of proteins and nutraceuticals, it is necessary to have a fast and online analysis technique, namely, in silico analysis methods.

In silico analysis is used to identify bioactive peptides from protein sequences using computational

methods, such as the use of databases, online applications, and software (Tu *et al*., 2018). This technique has been widely used to determine the potential of bioactive peptide compounds in fisheries and food products. One of the most commonly used protein databases is the National Center for Biotechnology Information (NCBI) database, which is used to obtain protein and amino acid profiles. Param Prot software was used to determine the composition and molecular weight of amino acids in the sequenced proteins. BIOPEP-UWM is an online application for estimating the content of bioactive peptides in protein sequences (Minkiewicz *et al*., 2019). These techniques are then used to observe the proteins and bioactive peptides of the mole crab protein hydrolysate, which will then be used for molecular docking to evaluate the interaction of the peptide with specific molecular targets, such as enzymes, receptors, or other proteins, and to determine the specific potential of anticancer, antioxidant, antibacterial, antihypertensive, antidiabetic, and anti-inflammatory agents (Tu *et al.*, 2018; Dinh & Agyei, 2021).

Some studies have reported that protein hydrolysates can be produced from several types of crabs, shrimps, and lobsters. Recent research has delved into the nutritional composition of mole crabs, particularly focusing on how processing methods influence the protein content. Maharani *et al*. (2022) observed that thermal processing, such as steaming, leads to protein hydrolysis in mole crabs, breaking down larger protein molecules into smaller peptides and amino acids. This process not only increases the protein concentration due to moisture loss but also enhances the digestibility and bioavailability of proteins. Furthermore, the enzymatic hydrolysis of crustacean proteins, including those from crabs, has been shown to produce protein hydrolysates with significant bioactive properties. These hydrolysates exhibit antioxidant activities and can serve as functional ingredients in food products. In the context of mole crabs, although specific studies on their protein hydrolysates are limited, general findings from related crustaceans suggest that *Emerita emeritus* could be a valuable source of bioactive peptides. These peptides have potential applications in developing functional foods aimed at promoting health benefits, such as antioxidant effects and improved protein intake

Mole crabs, including crustaceans, have not yet been published, making them the main raw material for producing protein hydrolysates. Therefore, this study aimed to evaluate the potential of a peptide derived from a mole crab protein hydrolysate using an in silico approach. The following is a visual representation of the research flowchart, as shown in Figure 1.

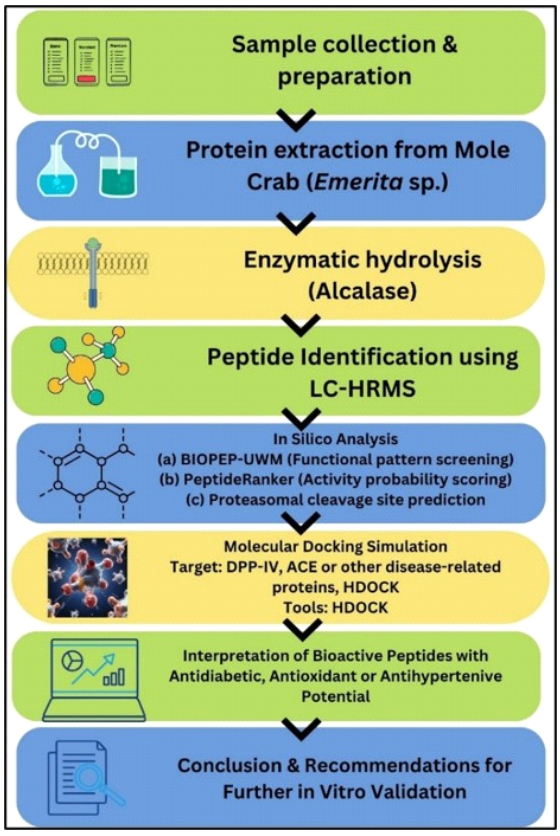


Figure 1. The Research Flow

Material

The raw material used was live mole crab (*Emerita* sp.) obtained from fishermen in the southern coastal area of Java, Yogyakarta, Indonesia. The samples were maintained in a cold chain using crushed ice at a 1:1 ratio and were transported to the laboratory within 3 h. Upon arrival, the samples were cleaned, washed, and frozen at "20/ °C until analysis, following standard handling protocols for crustaceans to preserve biochemical quality (Santoso *et al.*, 2015; Gokoglu & Yerlikaya, 2015).

Preparation of Fresh Mole Crab

The research process commences with the sampling of mole crabs, as illustrated in Figure 2. The mole crab (*Emerita* sp.) was prepared by washing with clean water to remove dirt and sediment that sticks and drains. A clean sample was chopped using a *grinder* to make it homogeneous and easy to decompose. Alcalase is a serine endopeptidase derived from *Bacillus licheniformis,* with optimal activity at pH 8–10 and temperatures between 50 and 60°C. It is widely used for protein hydrolysis owing to its broad specificity and high yield of bioactive peptides (Shaibani *et al*., 2020). Mole crabs are small crustaceans commonly found in sandy coastal areas with clean and oxygen-rich waters, preferring environments with salinity between 30 and 35 ppt, temperatures around 25–30/ °C, and pH levels of 7.5–8.5, which support their metabolic and reproductive processes (Haryati *et al*., 2022). Nutritional analysis of *Emerita* species has shown that they are rich in protein (up to 37.88%) and contain fat, ash, and bioactive components, such as carotenoids, which contribute to their antioxidant potential (Sandi *et al*., 2021). Moreover, the exoskeletons of mole crabs are a valuable source of chitin, which can be converted into chitosan. Chitosan derived from *Emerita emeritus* has been shown to exhibits promising biological activities, including antimicrobial, antioxidant, and anticoagulant properties, indicating its potential applications in food and pharmaceutical industries (Sukumaran *et al*., 2021).

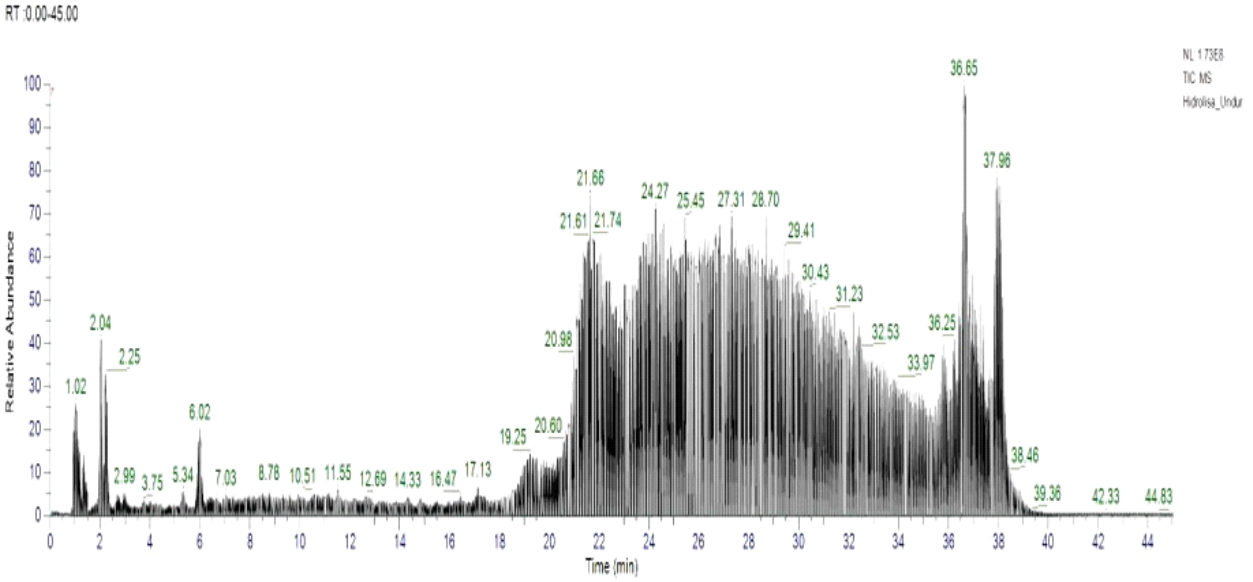


Figure 2. Total Ion Chromatography Graphic

Production of Mole Crab Protein Hydrolysate (Shaibani et al., 2020)

Mole chopped crabs were placed in a *glass beaker*. The chopped sample was then mixed with deionized water at a ratio (sample: deionized water) of (1:2), homogenized, and heated at 85°C for 20 min. Heating was performed to inactivate the endogenous enzymes. The sample was prepared by adding 1 N NaOH solution to create an alkaline atmosphere. The procedure was continued by hydrolysis by preparing Alcalase enzyme (Novozymes, Denmark) with an activity of 2.4 AU/g added to the sample at 2% concentration of the total sample weight. The hydrolysis was followed by heating at 55°C in a water bath for 120 min (the pH range 6 to 7). The samples that underwent hydrolysis were then heated at 95°C for 15 min to activate the alcalase enzyme, followed by centrifugation at 8000 rpm for 10 min. After centrifugation, the supernatant was collected, placed in a glass vial, and stored at -5°C for 1 d for further analysis.

Procedure Analyzes

Proteomics Sample Preparation for LC-HRMS (Windarsih et al., 2022)

A 500 mg sample was prepared and added to 15 ml of Tris-HCl buffer solution (pH 8.0) and then homogenized by *vortexing* for 1 min. The sample solution was sonicated at 50°C for 60 min and centrifuged at 7000 g for 6 min at room temperature. The centrifugation results in the form of supernatants were taken as 500 µL, and 500 µL of cold acetone solution (-20 °C) was added, followed by homogenization again with *vortexing* for 1 min. The sample solution was re-centrifuged at 10,000 × g for 5 min at a cold temperature (4°C). The supernatant was removed and centrifuged in *a vacuum concentrator* (Eppendorf) for 30 min at 60°C. The centrifuged samples were added to 500 µL of 50 mM Tris-HCl *Buffer*, 500 µL of 50 mM ammonium bicarbonate, and 4 µL of 50 mM *dithiothreitol*, homogenized for 1 min, and incubated at 75°C for 15 min. The samples were added to 9 ìL *of iodoacetamide and incubated* in a dark room for 30 min. The incubation results were added with 5 µL of 0.1 mg/mL solution of *Pierce Trypsin Protease MS grade* and vortex for 1 minute followed by incubation at room temperature for 17 hours. The incubation results were supplemented with carboxylic acid and filtered through 0.2 µL of PVDF. The solution was then injected into a *liquid chromatography-high-resolution mass spectrometry* (LC-HRMS) tool.

Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) Analysis

Protein sequences from marine retrograde hydrolysate peptides were analyzed using liquid chromatography-high-resolution mass spectrometry (LC-HRMS) (Thermo Scientific™ Orbitrap™ Explores 240 HRMS, Bremen, Germany) to obtain FASTA sequences from the mole crab protein hydrolysate for further analysis in silico. Liquid chromatography was performed using a Thermo Scientific™ Vanquish™ Horizon UHPLC system with a Binary Pump (Germering, Germany). Separation was achieved using a Thermo Scientific Acclaim PepMap 100 C18 column (150 mm × 1 mm ID, 3 µm particle size; Lithuania). The mobile phase consisted of MS-grade water containing 0.1% formic acid (solvent A) and MS-grade acetonitrile containing 0.1% formic acid (solvent B). The gradient was set as follows: 5% B for 1 min, a linear increase to 50% B over 30 min, held at 50% B for 2 min, increased to 90% B for 2 min, returned to 5% B, and held until the end of the 45-minute run. The flow rate was maintained at 75 µL/min, column temperature was set at 30 °C, and injection volume was 5 µL. High-resolution mass spectrometry (HRMS) analysis was conducted using a Thermo Scientific™ Orbitrap™ Exploris 240 (Bremen, Germany) equipped with an OptaMax™ NG Heated Electrospray Ionization (H-ESI) source. Data were acquired in the positive ion mode using a Full MS/dd-MS2 acquisition strategy. The full MS resolution was set at 120,000 FWHM, with a scan range of m/z 350–1,500, maximum injection time of 100 ms, and an intensity threshold of 8,000. Charge states 2–6 were selected, with a mass tolerance of 5 ppm. For dd-MS2, a resolution of 15,000 FWHM was used with a normalized collision energy (NCE) of 30, and nitrogen was employed as the collision gas. The source parameters were as follows: spray voltage, 4000 V; sheath gas, 10 arbitrary units (AU); auxiliary gas, 5 AU; sweep gas, 1 AU; ion transfer tube temperature, 300 °C.

In Silico Methods

The peptide sequence obtained from the LC-RHMS analysis was submitted to the Bioactive Peptide-University of Warmia and Mazury (BIOPEP-UWM) database website at https://biochemia.uwm.edu.pl/. This process was used to evaluate the potential active compounds within the sequence. The BIOPEP software is additionally used to determine the sensory peptide profile in this research.. The next step involved generating a 3D structure of the amino acids identified through biopeptide analysis for docking studies using University of California, San Fransisco (UCSF) Chimera V1.15 (2021). This software assists in constructing the 3D structure of amino acids by inputting them into a structure-editing menu. Subsequently, amino acid structures were minimized to reduce the energy required for docking. The minimized amino acid structures were then saved in the PDB file format. Ligand compounds to be tested were prepared, followed by the preparation of the target protein. The target protein structure was downloaded from the https://www.rcsb.org/ database in PDB format. The protein was cleaned by removing non-standard ligands and adding hydrogen atoms, and the modified protein structure was saved in the PDB format. Both the prepared ligand and the target protein were subjected to docking using the HADOCK server http://hdock.phys.hust.edu.cn/. This server facilitates docking between the peptide structures and target proteins. The prepared target protein and ligand were uploaded and the docking job was submitted. The results were sent to a registered email in the form of discovery file. Additionally, the server provided the output, including the binding affinity and RMSD values. Binding affinity represents the energy required for the ligand to bind to the target protein, whereas Root Mean

Square Deviation (RMSD) describes the conformational changes of the ligand during docking and its interaction with the target protein. After obtaining the results, the files were analyzed using Discovery Studio software 2020 to interpret the molecular docking outcomes.

Result and Discussion

Peptide Sequences from Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) Analysis

LC-HRMS analysis of peptides produced from mole crab protein hydrolysate (*Emerita* sp.) successfully identified several peptide sequences with potential bioactivities. Spectral data showed that the detected peptides had low to medium molecular masses with a typical fragmentation profile. Some identified peptides are listed in Table 1.

As shown in Table 1, 12 types of mole crab protein hydrolysate sequences were obtained from LC-HRMS analysis. The chromatographic profile obtained by LC-HRMS revealed a diverse distribution of peptides with a broad range of retention times and ion intensities, indicating the complexity of the hydrolysate mixture derived from *Emerita* sp. (Figure 2). The chromatogram displayed multiple peaks, indicating a complex mixture of peptide compounds eluted over a 45-minute gradient. Major peaks were observed at retention times of approximately 6.02, 21.66, 24.27, 28.70, 36.65, and 37.96 minutes, suggesting the presence of peptides with varying polarities and hydrophobicities. These peaks represent candidate bioactive peptides for further in silico and functional characterizations. The presence of multiple peaks suggests successful enzymatic hydrolysis, which generated a wide variety of peptide fragments. These findings are consistent with those of previous studies in which Iwaniak *et al*. (2016) confirmed that the identification of specific peptide signatures through chromatographic separation can serve as a preliminary step for selecting sequences with high nutraceutical potential. Therefore, the peptide mapping results in this study not only reflect successful hydrolysis but also support the presence of bioactive peptide candidates for further investigation. Another study by Gao *et al*. (2020) reported a similarly broad peptide distribution in shrimp protein hydrolysates, which was attributed to the efficiency of enzymatic digestion and complexity of marine proteins. Similarly, Shaibani *et al*. (2020) observed distinct chromatographic patterns in common carp hydrolysates that were linked to their antioxidant and antimicrobial properties. Windarsih *et al*. (2022) emphasized that the diversity of peptide peaks detected through LC-HRMS could be used to predict the potential bioactivity of hydrolysates, especially when paired with in silico analysis. Furthermore, Pearman *et al*. (2020) demonstrated that peptide distribution profiles obtained via LC-HRMS could correlate with functional classifications of peptides such as ACE-inhibitory or antimicrobial activity.

Table 1. Mole Crab Hydrolysate Peptide Sequence (*Emerita* sp.) from LC-HRMS Analysis

Protein	Accession	Peptida sequence
cytochrome c oxidase sub unit III (mitochondrion)	YP_010515280.1	MTSPHGHHAFHLVDMSPWPLTGSISAMMLTTGLVK
cytochrome oxidase sub unit I, partial	ACF75773.1	TSGMRLER
cytochrome c oxidase sub unit I, partial	QXP08892.1	HKDIGTLYFIFGAWAGMVGTSLSLIIR
NADH dehydrogenase sub unit 6 (mitochondrion)	UXL87270.1	MLYMTLPMIILISMMFIR
cytochrome c oxidase sub unit 1, partial	UXL87260.1	KESFGTLGMIYAMLAIGILGFIVWAHHMFTVGMDVDTR
NADH dehydrogenase sub unit 2 (mitochondrion)	UXL87263.1	KENIWVPLIIFFNFIGLMFPSMVI
histone H3, partial [***Emerita brasiliensis***]	AAZ39244.1	ARKSTGGK
NADH dehydrogenase sub unit 3 (mitochondrion)	UXL87262.1	KTIMDREK
NADH dehydrogenase sub unit 5 (mitochondrion)	UXL87267.1	KIIALSTLSQLGVMMSILALGFADLAFFHLLTHALFK
ATP synthase F0 sub unit 6 (mitochondrion)	YP_010515279.1	INYALHTEFK

cytochrome c oxidase subunit I, partial	**ADD15818.1**	**INYALHTEFK**
cytochrome b (mitochondrion)	YP_010515285.1	MMMPLRK

The peptides contained in the protein hydrolysate play important roles in metabolic processes and cellular functions, particularly in the electron transport chain, which contributes to cellular energy production. For example, mitochondrial peptides, such as humanin and MOTS-c, have been shown to enhance mitochondrial respiration and ATP-generating capacity, as well as promote mitochondrial biogenesis. Humanin has been reported to increase basal oxygen consumption rate, maximal respiration, and respiratory capacity, thereby improving ATP production in cells (Zhang *et al*., 2017). Moreover, the secondary structure of peptides, such as helical conformations, can influence electron transport efficiency. Research has shown that peptides with well-defined secondary structures, like beta-turns or 3 € helices, exhibit higher molecular conductance, indicating the importance of helical conformation in electron transport across peptides (Zhang *et al.*, 2024). Peptides produced from the hydrolysis of these proteins have potential in terms of bioactive activity as antioxidant, antihypertensive, anticancer, anti-inflammatory, antimicrobial, and immunomodulatory agents. One of the above types of protein peptide, cytochrome c oxidase subunit III (mitochondrion), is known to be involved in the oxidation-reduction process; therefore, peptides from this protein can play a role in counteracting oxidative stress. This statement has been revised based on scientific literature. According to Montinaro and Cicardi (2020), angiotensin-converting enzyme (ACE) inhibitors have been widely used in clinical practice to manage hypertension and other cardiovascular conditions owing to their proven efficacy in reducing blood pressure and protecting against organ damage. These inhibitors block the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, thereby promoting vasodilation and lowering the systemic blood pressure.

In addition, because it is at the top, the protein peptide will be further analyzed in terms of its potential. The identification of these peptides supports the possibility that mole crab protein hydrolysates contain bioactive components relevant for applications in functional foods or pharmaceuticals. Previous studies have identified peptides derived from marine protein hydrolysates using bioinformatic tools. For example, Tu *et al*. (2018) highlighted the importance of bioinformatics tools for identifying and predicting peptide bioactivity and for improving the efficiency of functional peptide discovery. Evidence of diverse peptide profiles of shrimp shell hydrolysates (Gao *et al*., 2020) and antimicrobial and antioxidant activities of common carp muscle protein hydrolysates (Shaibani *et al*., 2020) supports the versatility of marine-derived peptides for nutraceutical development. Similarly, Cassano *et al*. (2021) demonstrated that specific peptides obtained from fish skin hydrolysates exhibit potent antioxidant and anti-inflammatory properties, contributing to their functional food applications. Recently, Bauer *et al.* (2024) emphasized the therapeutic potential of bioactive peptides from crustacean sources in the management of chronic diseases such as hypertension and diabetes. These findings validate our results and strengthen the potential of *Emerita* sp. hydrolysate as a promising source of bioactive peptides.

*Mole Crab Hydrolysate Peptide Activity (***Emerita *sp.)**

The bioactive activity of the mole crab hydrolysate was analyzed using Peptide Ranker according to the protocol described by Mooney *et al*. (2013) and BIOPEP-UWM (Minkiewicz *et al*., 2019) software. BIOPEP is a bioinformatic software designed to analyze and predict the bioactive potential of peptides from protein sequences. This software provides database information in the form of proteins, bioactive peptides, allergenic proteins with epitopes, sensory peptides, and amino acids. Structural prediction was performed using UCSF Chimera v1.15 (2021), and docking was conducted using the HDOCK server (2022) with visualization in Discovery Studio 2020. The activities of the moles of crab hydrolysate are presented in Table 2.

Table 2. Activity Potential Profile of Peptides from Mole Crab Hydrolysate (*Emerita* sp.)

Peptide ID	Sequence	Location	Name	Activity	Monoisotopic mass	Chemical mass
3317	HL	[11-12]	Antioxidative peptide	antioxidative	268,1531	268,3114
7513	PL	[19-20]	ACE inhibitor from Alaskan pollack skin	ACE inhibitor	228,1469	228,2873
7558	VK	[34-35]	ACE inhibitor from buckwheat	ACE inhibitor	245,1734	245,3177
7602	HL	[11-12]	ACE inhibitor	ACE inhibitor	268,1531	268,3114
8190	PW	[17-18]	peptide from buckwheat	antioxidative	301,1422	301,3396
8557	HL	[11-12]	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	dipeptidyl peptidase IV inhibitor	268,1531	268,3114
8638	PL	[19-20]	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	dipeptidyl peptidase IV inhibitor	228,1469	228,2873
8865	PW	[17-18]	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	dipeptidyl peptidase IV inhibitor	301,1422	
8921	VK	[34-35]	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	dipeptidyl peptidase IV inhibitor	245,1734	245,3177
9493	HL	[11-12]	DPP-III inhibitor	dipeptidyl peptidase III inhibitor	268,1531	268,3114
10462	PL	[19-20]	Inhibitor of Xaa-Pro aminopeptidase	xaa-pro inhibitor	228,1469	228,2873
10463	PL	[19-20]	Inhibitor of Lactocepin	lactocepin inhibitor	228,1469	228,2873

H(His)-Histidine, L(Leu)-Leucine, P(Pro)-Proline, V(Val)-Valine, K(Lys)-Lysine, W(Trp)-Tryptophan

The activity of the bioactive peptide from the mole crab hydrolysate (Table) 2 was obtained from the MTSPHGHHAFHLVDMSPWPLTGSISAMMLTTGLVK sequence derived from the cytochrome c oxidase protein subunit III (mitochondrion). Each peptide analyzed had different activities. The peptide activities are shown in (Table 2.) Twelve bioactive peptide sequences were analyzed, which showed the enzymatic activity profile of the marine hydrolysate. Although Table 2 lists dipeptides (e.g., PW and AF), these represent functional motifs embedded within longer peptide sequences identified by LC-HRMS. For instance, the peptide “MTSPHGHHAFHLVDM SPWPLTGSISAMMLTTGLVK” contains the motif “PW,” which is known for its strong antioxidant and ACE inhibitory activity (Tu *et al.,* 2018). As shown in Table 2, there are six groups of peptide activities dominated by two large groups: ACE inhibitors (ACEi) and DPPIV inhibitors. ACEi have antihypertensive effects and provide organ protection in common clinical conditions, such as diabetes mellitus (Montinaro & Cicardi, 2020). DPPIV inhibitors inhibit incretin degradation in patients with type 2 diabetes mellitus (Cassano *et al*. 2021). Subgroups of peptide activities include antioxidants, xaa-pro inhibitors, and lactocepin inhibitors. The highest monoisotope mass was obtained in the PW bioactive peptide sequence with a value of 301.1422, which acts as a DPPIV inhibitor and an antioxidant. The lowest monoisotope mass was obtained for the PL bioactive peptide sequence with a value of 228.1469, which acts as an ACE, DPPIV, xaa-pro, and lactocepin inhibitor. All activities obtained were derived from the dipeptide class. There was little difference between the monoisotope and chemical mass. These combined approaches, database matching, machine learning-based prediction, and structural binding validation, provide a scientifically supported basis for identifying peptide sequences with bioactive potential.

BIOPEP can also be used to analyze the sensory properties of the obtained peptides. The sensory peptides from the mole crab hydrolysate (*Emerita* sp.) are shown in Table 3

As shown in Table 3, 12 types of peptides were analyzed using BIOPEP, which contributed to the sensory properties of the mole crab hydrolysate (*Emerita* sp.). Peptides play biological roles and influence the taste of a product. Sensory peptides that affect taste have been the subject of research for the past 50 years because of their taste-altering properties, including in silico studies of bitter taste (Iwaniak *et al*., 2016). The number of sensory identifications varied, indicating that several peptides contributed to the sensory profile. Sensory tastes can be classified into bitterness and sweetness. Each group had different tastes. Sensory peptide taste was

dominated by bitter taste, with 10 types of peptides contributing, and the least sensory taste was sweet taste, with two types of peptides contributing. The ranking of moles of the crab hydrolysate peptides (*Emerita* sp.) is shown in Table 4.

Table 3. Sensory Properties of Peptides from Mole Crab Hydrolysate (*Emerita* sp.)

Peptide ID	Bioactive Sequence	Location	Name	Activity
3317	HL	[11-12]	Bitter amino acid	Bitter
7513	PL	[19-20]	Bitter amino acid	Bitter
7558	VK	[34-35]	Bitter peptide	Bitter
7602	HL	[11-12]	Bitter amino acid	Bitter
8190	PW	[17-18]	Sweet amino acid	Sweet
8557	HL	[11-12]	Bitter amino acid	Bitter
8638	PL	[19-20]	Bitter amino acid	Bitter
8865	PW	[17-18]	Sweet amino acid	Sweet
8921	VK	[34-35]	Bitter peptide	Bitter
9493	HL	[11-12]	Bitter amino acid	Bitter
10462	PL	[19-20]	Bitter amino acid	Bitter
10463	PL	[19-20]	Bitter amino acid	Bitter

H(His)-Histidine, L(Leu)-Leucine, P(Pro)-Proline, V(Val)-Valine, K(Lys)-Lysine, W(Trp)-Tryptophan

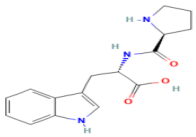
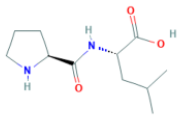
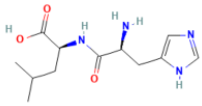
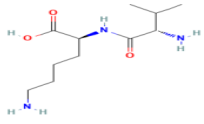
Table 4. Peptide Rank of Mole Crab Hydrolysate (*Emerita* sp.)

Peptide Rank	Bioactive Sequence
0.992911	PW
0.811148	PL
0.374865	HL
0.03329	VK

H(His)-Histidine, L(Leu)-Leucine, P(Pro)-Proline, V(Val)-Valine, K(Lys)-Lysine, W(Trp)-Tryptophan

Based on Table 4, it can be seen that there are 4 levels of bioactive peptides found in the hydrolysate of the sea retreat, namely 2 PW, 4 PL, 4 HL, and 1 VK. The lowest value was obtained for the VK sequence (0.03329), whereas the highest was obtained for the PW sequence (0.992911). Bioactive peptides were identified using the Peptide Ranker tool (Pearman *et al*., 2020). The peptide rating value indicates the probability of bioactivity of each peptide. Higher-ranked peptide scores indicate higher bioactive peptide content. The threshold value for peptide bioactivity was set to 0.5. Higher values indicate that the peptide has strong bioactivity, whereas lower values (especially below the threshold of 0.5) indicate that the bioactivity of the peptide is low or non-existent (Mooney *et al*., 2013). The identification of the peptide chemical profiles is presented in Table 5.

Table 5. Identification of Chemical Profile of Mole Crab Hydrolysate Peptide (*Emerita* sp.)

Peptide	Formula	Name	Canonical SMILES	2D Structure
PW	$C_{16}H_{19}N_3O_3$	L-prolyl-L-tryptophan	C1C[C@H](NC1)C(=O)N[C@@H](CC2=CNC3=CC=CC=C32)C(=O)O	
PL	$C_{11}H_{20}N_2O_3$	L-prolyl-L-leucine	CC(C)C[C@@H](C(=O)O)NC(=O)[C@@H]1CCCN1	
HL	$C_{12}H_{20}N_4O_3$	L-histidyl-L-leucine	CC(C)C[C@@H](C(=O)O)NC(=O)[C@H](CC1=CN=CN1)N	
VK	$C_{11}H_{23}N_3O_3$	L-valyl-L-lysine	CC(C)[C@@H](C(=O)N[C@@H](CCCCN)C(=O)O)N	

H(His)-Histidine, L(Leu)-Leucine, P(Pro)-Proline, V(Val)-Valine, K(Lys)-Lysine, W(Trp)-Tryptophan

Molecular Docking

Molecular docking is a technique used in chemical computing to predict the interactions between two molecules, particularly ligands (such as peptides, drugs, or other bioactive compounds) and protein targets (such as enzymes or receptors). This process aims to determine the best orientation and position of the ligand molecule when it binds to a molecular target, and to predict the strength and stability of such interactions. Molecular docking has been used to study molecular interactions in the context of medicine, biotechnology, and the development of bioactive compounds.

Table 6. Analysis of Molecular Docking from Mole Crab Hydrolysate (*Emerita* sp.)

Protein	Sequence	Activity	PDB ID (Reseptor)	Binding Affinity	Confidence Score	RMSD
Cytochrome c oxidase subunit III (mitochondrion)	PL	ACE inhibitor	1O86	-107,37	0,29	36,97
	VK	ACE inhibitor	1O86	-103,72	0,28	37,36
	AF	ACE inhibitor	1O86	-129,70	0,39	69,62
	GH	ACE inhibitor	1O86	-121,35	0,36	35,25
	PL	DPP IV inhibitor	3G0E	-89,73	0,23	126,15
	AF	DPP IV inhibitor	3G0E	-108,43	0,3	90,41
	GH	DPP IV inhibitor	3G0E	-113,68	0,32	127,24
	VK	DPP IV inhibitor	3G0E	-91,07	0,23	127,35
	AF	inhibitor of tripeptidyl peptidase II	6B98	-113,94	0,32	37,34

H(His)-Histidine, L(Leu)-Leucine, P(Pro)-Proline, V(Val)-Valine, K(Lys)-Lysine, W(Trp)-Tryptophan

Marine product peptides have promising multitarget capabilities in the development of therapies for multifactorial diseases, including hypertension, owing to their diverse bioactivities such as antihypertensive, antioxidant, and anticancer activities. Based on Table 6, the results of molecular docking analysis of the mole crab hydrolysate peptide (*Emerita* sp.) show that the AF peptide has the strongest binding potential to the molecular target of Angiotensin-Converting Enzyme (ACE), with a binding affinity value of -129.70. This shows that AF is a potential candidate ACE inhibitor for the management of hypertension. In addition, the GH peptide showed significant interaction with ACE, with a binding affinity of -121.35. Regarding the inhibitory activity of Dipeptidyl Peptidase IV (DPP-IV), GH peptide had the best binding affinity value of -113.68, followed by AF with a value of -108.43. Both peptides showed strong potential for development as type 2 anti-diabetic compounds. AF peptides also have a good binding ability to Tripeptidyl Peptidase II, with a binding affinity value of -113.94, which indicates the possibility of additional activity against the target. Other peptides, such as PL and VK, have higher binding affinities, namely -107.37 and -103.72 against ACE, and -89.73 and -91.07 DPP-IV, (respectively); therefore, their activity is relatively weaker than that of AF and GH. These results indicate that AF and GH peptides have superior bioactivity compared to other peptides of the same protein. With a supportive confidence score and RMSD values, the results of this molecular docking study showed that peptides from hydrolysates of marine retreat proteins, especially AF and GH, have the potential to be further developed as bioactive compounds for the treatment of hypertension and type 2 diabetes (Bauer *et al*., 2024).

Conclusion

This study shows that, based on the results of in silico analysis, peptides produced from the hydrolysate of the marine retrograde protein (*Emerita* sp.) show significant bioactivity potential. From the analysis, 12 peptide sequences were identified, most of which originated from mitochondrial proteins such as cytochrome c oxidase and NADH dehydrogenase. Several of these peptides, including MTSPHGHHAFHLVDMSPWPLTGSISAMMLTTGLVK and INYALHTEFK, showed potential bioactivities, such as ACE inhibition, DPP-IV inhibition, and antioxidant and anti-inflammatory effects. The dipeptide Pro-Trp (PW) had the highest bioactivity prediction score (0.993), suggesting its strong

potential as an antioxidant and antidiabetic agent. Molecular docking analysis further supported these findings, with the AF peptide showing the best interaction against ACE (-129.70 kcal/mol) and the GH peptide demonstrating strong binding to DPP-IV (-113.68 kcal/mol).

The results show that the identified peptides could be good candidates for making functional foods or supplements. These foods could help with heart health, diabetes, and act as antioxidants. The study highlights the importance of marine resources, especially less-used species like *Emerita* sp., in finding new bioactive compounds that could have health benefits.

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