Research Article

Valorization of Jellyfish (*Rhopilema hispidum*) By-Products for Bioactive Peptides with Antibacterial, Enzyme Inhibitory, and Low Cytotoxic Activities

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Abstract
The escalating concern regarding antibiotic resistance and metabolic disorders has catalyzed the search for natural compounds with multifunctional bioactivities. Marine-derived peptides have surfaced as promising candidates due to their diverse structures and bioactive properties. This study investigates the enzymatic hydrolysis of low-cost salted jellyfish (*Rhopilema hispidum*) by-products using pepsin to produce bioactive peptides with multifunctional attributes. The resulting hydrolysates were purified through reverse-phase and ion exchange chromatography and assessed for their antibacterial activity against *Escherichia coli*, *Vibrio parahaemolyticus*, and *Staphylococcus aureus*. Among the synthesized peptides, NQKAMQELNE exhibited significant antibacterial effects against *E. coli* (28.95%) and *S. aureus* (51.93%) and demonstrated substantial inhibitory actions on α-amylase (100.00%) and α-glucosidase (46.99%). Additionally, PFTMYFLL displayed remarkable inhibitory activity against *V. parahaemolyticus* (42.88%). Importantly, all five synthesized peptides—NQKAMQELNE, TDSPAPSETTD, EQIYPMGEGDEL, PFTMYFLL, and PMETDDQPNN—exhibited low hemolytic activity (4.14–7.12%), indicating minimal cytotoxicity and a favorable safety profile. Mechanistic insights suggest that the antibacterial effects of these peptides may arise from their capacity to disrupt vital intracellular microbial processes. This research addresses environmental and economic challenges by valorizing underutilized marine by-products, thereby contributing to developing safe, natural, and multifunctional bioactive compounds. These findings highlight the potential of jellyfish-derived peptides as functional ingredients in the food and pharmaceutical industries.

Keywords: Antibacterial activity, Jellyfish (*Rhopilema hispidum*) by-products, Peptides, Protein hydrolysate

1 Introduction

Health plays a vital role in our lives, significantly impacting our relationships and professional effectiveness. Consuming nutritious foods is essential for maintaining good health and offers protection against chronic diseases. However, foodborne illnesses, which occur due to the ingestion of food contaminated with pathogenic microorganisms and their toxins, have been well-documented. These illnesses can result in increased hospitalizations and, in some instances, fatalities [1]. According to the World Health Organization (WHO), there are 600 million reported cases of foodborne diseases worldwide, affecting approximately 1 in 13 people in the global population, with around 420,000 deaths each year [2]. Bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus* are the primary agents responsible for these diseases [3], [4]. While antibiotics are frequently prescribed to treat infections, the growing issue of antimicrobial resistance (AMR), driven by the overuse of these medications, has emerged as a significant concern [5]. WHO reports emphasize the urgent need for action to prevent an antimicrobial resistance crisis and underscore the necessity of discovering and developing new alternative antibiotics [6].

Antibiotics can be derived from various sources, with natural microorganisms being a notable one. These microorganisms produce substances to combat competing organisms in their environment [7]. Some antibiotics are developed by modifying or chemically altering the core structures of these naturally occurring compounds, thereby enhancing their properties—such as broadening their spectrum of activity, improving their resistance to degradation within the body, or reducing side effects [8]. Additionally, certain antibiotics are entirely synthesized in laboratories, independent of microorganisms [8]. These synthetic antibiotics are often designed with specific structures aimed at targeting bacterial pathogens. This pioneering research has the potential to unlock transformative solutions to modern health challenges, making it a particularly intriguing field of study.

Antimicrobial peptides (AMPs) represent an innovative class of antibiotic agents that offer significant advantages over traditional antibiotics. With their broad-spectrum antimicrobial activity, these potent peptides effectively kill or inhibit harmful pathogens, including bacteria, fungi, and certain viruses [9]. What sets AMPs apart is their unique mechanism of action, which involves disrupting or destroying the structural integrity and membranes of microbial cells. This approach significantly complicates the ability of pathogens to develop resistance, leading to a reduced likelihood of resistance compared to conventional antibiotics [9]. Furthermore, many AMPs exhibit low toxicity to human cells, particularly those derived from the diverse marine proteins [10]. For example, Cod-derived AMPs demonstrate potent activity against Gram-negative bacteria and fungi, but exhibit minimal efficacy against Gram-positive bacteria. Conversely, AMPs isolated from blind eels display broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria, while lacking antifungal properties [10]. In addition to their remarkable antibacterial properties, certain peptides display intriguing antioxidant, anti-inflammatory, and enzyme-inhibitory activities [11]–[14]. These peptides can inhibit crucial enzymes such as angiotensin-I-converting enzyme or α-glucosidase and α-amylase—key players in the conversion of carbohydrates into glucose. This regulatory function is vital, as elevated blood sugar levels are a significant contributor to the development of diabetes [15], [16]. While pathogens like *E. coli*, *S. aureus*, and *V. parahaemolyticus* may not directly lead to diabetes, they are linked to an increased risk of infections and complications in diabetic patients [17]. Therefore, the quest for novel peptides derived from natural protein sources—particularly those that effectively integrate antimicrobial properties with enzyme inhibition and low cytotoxicity—has gained considerable interest.

The development of functional foods and pharmaceutical agents has become an increasingly compelling area of interest. This study focuses on by-products from sand jellyfish (*Rhopilema hispidum*), which are waste materials generated during the production of salted jellyfish for export [13]. These by-products serve as a source of marine protein for peptide production. In contrast to other marine sources like fish or shrimp, jellyfish—especially *R. hispidum*—thrive abundantly in some areas of Asia and generate substantial amounts of processing waste while producing salted jellyfish products. Remarkably, these by-products, often discarded without a second thought, are treasure troves of collagen and contain uniquely structured proteins highly amenable to enzymatic hydrolysis, yielding bioactive peptides. Furthermore, the lower lipid content in jellyfish helps to minimize unwanted interactions during the purification process of these peptides [11], [13], [18]. As a result, *R. hispidum*

stands out as a promising, sustainable, and economical protein source for creating bioactive peptides with multifunctional benefits, inviting us to explore its untapped potential. Previous research has suggested that the by-products of sand jellyfish are promising for generating peptides with ACE-inhibitory, antioxidant, and anti-inflammatory activities [11]. However, to date, there have been no studies examining the antibacterial activity of peptides derived from purified salted sand jellyfish by-products through reverse-phase and ion exchange chromatography, nor their effects on the enzymes α-amylase and α-glucosidase. This study aimed to investigate the antibacterial activity of purified peptides derived from sand jellyfish, identify potent antibacterial peptides, synthesize selected peptides, and evaluate their antibacterial properties, along with their inhibitory effects on α-glucosidase and α-amylase, and their hemolytic activity. Given their diverse bioactivities and low cytotoxicity, the findings of this research might lead to improved, safer, and more effective therapeutic strategies for treating infections, particularly those commonly encountered in diabetic patients.

2 Materials and Methods

2.1 *Jellyfish protein hydrolysate preparation*

Jellyfish protein hydrolysate was prepared using methods adapted from previous studies [11]–[13], [19], [20] with minor modifications. In summary, the salted by-products of jellyfish (*R. hispidum*), specifically the umbrella and oral arms, were washed to remove salt. Subsequently, desalted samples were dried at 60 °C using a tray dryer (ED 400, Binder, USA) for 24 h. The dried samples were ground into a powder with a particle size of 100 mesh. Next, the jellyfish protein powder was heated at 95 °C in sodium acetate buffer (0.05 M, pH 4), with a ratio of jellyfish protein powder to sodium acetate buffer of 1:25 (w/v). The pepsin was added to the mixture (enzyme: substrate at ratio of 3:20 (w/w)) to initiate enzymatic hydrolysis. Hydrolysis was performed by shaking the mixture (150 rpm) at 37 °C for 48 h. The reaction was terminated by heating the mixture for 10 min. Afterward, it was centrifuged for 30 min at 9500 x g and filtered using filter paper (Whatman No. 1). The hydrolysate was retained at –18 °C until further analysis.

2.2 *Purification of peptides*

Reversed-phase and ion exchange chromatography (cation and anion) were used for purification, following a previously established method [11]. Initially, the hydrolysate was introduced into a C18 column (Amberlite® XAD®-2). Subsequently, formic acid (0.1%) and sterile water were used to wash the column to eliminate impurities, and the protein was eluted using 100% acetonitrile. Evaporation of the eluent was carried out at 50 °C. The dried sample was reconstituted in a 10 mM sodium acetate buffer (pH 4.0) and then subjected to a C18 column for stepwise elution using a gradient of acetonitrile at the following concentrations: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. Antibacterial activity was assessed for all fractions, with those exhibiting activity undergoing further purification via cation (SP Sepharose™) and anion exchange chromatography (Q Sepharose™). 1 M sodium chloride (NaCl) was utilized to elute the peptide from the columns. Subsequently, the eluent was loaded onto the C18 column to remove the NaCl, again using 100% acetonitrile as the eluent. The acetonitrile was evaporated in a hot air oven at 50 °C. After this step, the sample was dissolved in a 10 mM sodium acetate buffer at pH 4.0, and it was analyzed for soluble peptide content and antibacterial activity against *E. coli*, *V. parahaemolyticus*, and *S. aureus*.

2.3 *Peptide synthesis*

Peptides were synthesized by GenScript Biotech Inc. in New Jersey, USA, following the solid-phase synthesis with the fluorenylmethoxycarbonyl (Fmoc) method [21]. The purity of the synthesized peptides was higher than 85%, which was evaluated by high-performance liquid chromatography (HPLC) analysis.

2.4 *Analysis*

2.4.1 Determination of soluble protein content

All samples' soluble protein content was determined using the Lowry method [22]. Bovine serum albumin (BSA) was used as the standard for comparison.

2.4.2 Antibacterial activity assay

Three pathogenic bacteria were selected for determination: *E. coli* (ATCC 25922), *V. parahaemolyticus* (ATCC 17802), and *S. aureus*

(ATCC 25923). The antibacterial activity of hydrolysate and peptide samples was analyzed using the methods established by Muangrod *et. al*., [13] and Ditsawanon *et al*. [23], with minor modifications. Each bacterial strain was cultured on tryptic soy agar (TSA) and incubated for 24 h at 37 °C. Then, a colony from each bacterial strain was added to tryptic soy broth (TSB) and agitated at 200 rpm for 12 to 16 h at 37 °C to achieve a starter culture with an optical density (OD) of 0.05 at 600 nm. Kanamycin, an antibiotic, was utilized as a positive control. A volume of 50 µL (0.2 µg/µL) of the test samples (jellyfish protein hydrolysate, peptide, and antibiotic) was pipetted into 96-well microtiter plates, followed by the addition of 50 µL of the starter culture to each well. The plates were then agitated at 200 rpm for 6 h at 37 °C. After incubation, the OD600 values were measured using a microplate reader. The antibacterial activity (percentage inhibition) was calculated according to Equation (1):

$$\%Inhibition = \left(\frac{OD_{control} - OD_{sample}}{OD_{control}} \right) \times 100 \quad (1)$$

where $OD_{control}$ is the control's absorbance, which includes all reagents except the test samples, and OD_{sample} is the sample's absorbance (jellyfish protein hydrolysate, peptide, and antibiotic) with reagents added.

2.4.3 Anti-α-amylase activity assay

The assay was conducted using α-amylase (0.2 U/mL) and ethylidene-pNP-G7 (0.5 mM) in a 100 mM phosphate buffer (pH 6.8). 5 µL of the sample was combined with 45 µL of α-amylase enzyme and incubated at 37 °C for 10 min. Following this, 50 µL of ethylidene-pNP-G7 was added, and the reactions were measured at 405 nm for 15 min at 37 °C using a microplate reader. The results were presented as %inhibition, calculated using Equation (2):

$$\%Inhibition = \left(\frac{1-(B-b)}{(A-a)} \right) \times 100 \quad (2)$$

where A is the control's absorbance with enzyme (control), a is the control's absorbance without enzyme (control blank), B is the sample's absorbance with enzyme (sample), and b is the sample's absorbance without enzyme (sample blank).

2.4.4 Anti-α-glucosidase activity assay

The assay for α-glucosidase was performed at a concentration of 0.2 U/mL, utilizing p-nitrophenyl-α-D-glucopyranoside (pNPG) at 0.5 mM, also in 100 mM phosphate buffer (pH 6.8). A 5 µL sample was mixed with 45 µL of the α-glucosidase and incubated for 10 min at 37 °C. After adding 50 µL of pNPG, the reactions were monitored at 405 nm for 15 min at 37 °C using a microplate reader. The results were calculated according to Equation (2):

2.4.5 Hemolysis assay

The hemolytic activity of the test peptides was evaluated using a modified method based on Teerapo *et al*. [24]. In summary, 50 µL of a peptide solution (100 µg/mL) was combined with 50 µL of a 2% suspension of red blood cells in phosphate-buffered saline (PBS). This mixture was incubated for 10 min at 37°C, followed by centrifugation at 1500 rpm for 2 min. The supernatant was then carefully loaded into a 96-well microtiter plate, and the absorbance was monitored at 405 nm using a microplate reader. PBS and 1% SDS served as positive and negative controls, respectively. The hemolytic activity (%hemolysis) was calculated using Equation (3):

$$\%Hemolysis = \left(\frac{OD_{sample} - OD_{blank}}{OD_{1\%SDS} - OD_{blank}} \right) \times 100 \quad (3)$$

where OD_{sample} is the absorbance of the supernatant from the mixture of the peptide solution and red blood cell suspension, OD_{blank} is the absorbance of PBS, and $OD_{1\%SDS}$ is the absorbance of the supernatant from the mix of 1% SDS and red blood cell suspension.

2.4.6 Investigation of the interaction mechanism between peptides and microorganisms

The pathogenic bacteria (*E. coli*, *V. parahaemolyticus*, and *S. aureus*) were treated with selected synthetic peptides for 6 h. Then, the treated sample (with peptide and antibiotic) and the untreated sample (control) were digested with trypsin. After that, the protein profile of these samples was analyzed by LC-MS/MS using the Ultimate 3000 Nano/Capillary LC System (Thermo Scientific, UK) coupled to a Hybrid quadrupole Q-Tof impact II™ (Bruker Daltonics)

equipped with a Nano-captive spray ion source. Protein quantification of each sample was performed using MaxQuant 2.5.0.0 [25], with the Andromeda search engine employed to match MS/MS spectra against the UniProt *E. coli*, *V. parahaemolyticus*, and *S. aureus* databases [25]. A Venn diagram was created to illustrate the similarities and differences in protein expression patterns observed under the various treatments [26], with protein function annotations sourced from UniProt (https://www.uniprot.org/id-mapping).

2.5 *Statistical analysis*

All experiments were performed in triplicate. The results were presented as mean values with standard deviations. Sample variance was assessed using the SPSS 22.0 for Windows (SPSS Inc, Chicago, IL, USA). Duncan's multiple range test was chosen for post-hoc comparison to evaluate significant differences among the treatment groups (p-value < 0.05).

3 Results and Discussions

3.1 *Antibacterial activity of jellyfish protein hydrolysate*

The protein hydrolysate obtained from salted jellyfish's umbrella (PU) and oral arms (PO) has soluble protein contents of 14.47 ± 0.07 mg/mL and 13.62 ± 0.20 mg/mL, respectively. A soluble protein content of 0.2 mg/mL was used for the antibacterial activity test. The antibacterial effects of the jellyfish protein hydrolysate against *E. coli*, *V. parahaemolyticus*, and *S. aureus* are summarized in Table 1. Of the three tested pathogens, *V. parahaemolyticus* was found to be the most susceptible strain, particularly when treated with the PU sample, which showed the highest inhibition (17.23 ± 0.57%).

Table 1: The antibacterial activity of jellyfish protein hydrolysate.

Samples	Antibacterial Activity (%inhibition)		
	E. coli*	***V. parahaemolyticus****	***S. aureus****
Kanamycin	82.71±0.27^{A}	78.53±0.25^{A}	70.24±0.87^{A}
PU	14.79±1.36^{B}	17.23±0.57^{B}	16.28±0.89^{B}
PO	8.87±0.76^{C}	8.26±0.87^{C}	13.42±0.32^{C}

*Within each column, values marked with different superscript letters (A-C) reflect significant variation at the p-value < 0.05 level.

PU showed relatively higher activity than PO in all strains. The stronger antibacterial activity observed in the PU may be attributed to differences in protein profile between the umbrella and oral arms of the jellyfish [11], [27]. The umbrella region may harbor proteins that are more susceptible to enzymatic hydrolysis into potent antimicrobial peptides, possibly due to differences in amino acid composition or structural conformation [11]. Notably, both PU and PO demonstrated relatively higher inhibition against *S. aureus* compared to *E. coli*, especially in the PO sample, which showed the lowest effect on *V. parahaemolyticus* but moderate activity on *S. aureus*. These findings indicate that the antibacterial potential varies depending on the bacterial type and the source of the hydrolysate. The results of this study exceed those of previous research [13], which reported that jellyfish protein hydrolysate, treated with 5% pepsin for 24 h, displayed an inhibitory effect against *V. parahaemolyticus* ranging from 8.31% to 10.53%. Nonetheless, the antibacterial activity of jellyfish protein hydrolysate is still relatively low compared to antibiotics such as Kanamycin at equivalent concentrations. As a result, purification methods are recommended to isolate peptides with enhanced efficacy, thereby improving antibacterial activity.

3.2 *Antibacterial activity of purified peptides*

The jellyfish protein hydrolysates, identified as PU and PO, were purified through reversed-phase chromatography, resulting in the designation of the purified hydrolysates as PUR and POR. The percent inhibition of PUR and POR against *E. coli* was 18.38 ± 0.68% and 11.49 ± 0.26%, respectively. Additionally, the inhibition percentages against *V. parahaemolyticus* were recorded at 20.37 ± 0.64% for PUR and 10.77 ± 0.50% for POR. Furthermore, PUR exhibited a percent inhibition of 17.19 ± 0.49% against *S. aureus*, while POR showed a value of 14.50±0.40%. These results indicate that both PUR and POR displayed higher antibacterial activities against all three tested bacterial strains compared to their respective unpurified forms, PU and PO. The enhanced antibacterial effects observed in PUR and POR can be attributed to the purification process via reversed-phase chromatography, which selectively enriches hydrophobic and bioactive peptides. These findings are consistent with previous studies suggesting that most antimicrobial or antibacterial peptides typically exhibit amphipathic structures and contain a high proportion (often around 50%) of

hydrophobic amino acids [28]. The purification process likely concentrates peptides with greater membrane-disruptive potential. Supporting this, Tan *et al*., [29] demonstrated that synthetic peptides with increased hydrophobicity, achieved through substitution with tryptophan (W), exhibited improved antibacterial activity. Therefore, the higher activity of PUR and POR may be due to the presence of more potent, hydrophobic antibacterial peptides that were effectively isolated during purification. Subsequently, PUR and POR underwent gradient purification. Following this process and using acetonitrile concentrations ranging from 10% to 100%, the samples were renamed PUR10-PUR100 and POR10-POR100. The results revealed that for the acetonitrile concentration increase from 10% to 50%, the soluble protein contents of PUR10-PUR50 were measured at 1.04 ± 0.01, 2.12 ± 0.02, 1.41 ± 0.03, 0.36 ± 0.01, and 0.01 ± 0.01 mg/mL, respectively. The soluble protein contents for POR10-POR50 were recorded as 0.93 ± 0.01, 2.09 ± 0.01, 1.36 ± 0.01, 0.39 ± 0.01, and 0.04 ± 0.01 mg/mL, respectively. The peptide fractions (PUR10-30 and POR10-30) were analyzed for antibacterial activity due to their significant soluble protein concentrations, which were deemed sufficient for further investigation.

The antibacterial activity of PUR and POR, following purification via reversed-phase chromatography and elution with varying acetonitrile concentrations (10–30%), is presented in Figure 1. The jellyfish peptide fractions eluted at these concentrations were labeled PUR10, POR10, PUR20, POR20, PUR30, and POR30. The peptide fractions eluted with 20% acetonitrile exhibited the highest antibacterial activity among all tested fractions.

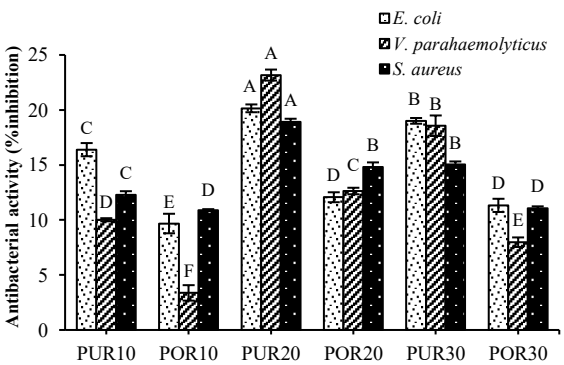

Figure 1: The antibacterial activity of jellyfish peptide after purification by reversed-phase chromatography (eluent = 10–30% acetonitrile). Bars labeled with different letters within the same bacteria exhibit statistically significant differences (p-value < 0.05).

These fractions demonstrated significant inhibitory effects against *E. coli*, *V. parahaemolyticus*, and *S. aureus*. In particular, the antibacterial activity against *E. coli* and *V. parahaemolyticus* increased by approximately 1.34 to 1.52 times and by 1.10 to 1.16 times against *S. aureus* compared to the jellyfish protein hydrolysate before purification. While hydrophobicity plays a role in the antibacterial effectiveness of a peptide, other characteristics such as structure, amino acid sequence, and even charge can also influence its antibacterial capabilities [28], [30], [31]. Therefore, PUR20 and POR20 were selected for further purification.

PUR20 and POR20 were purified using cation and anion exchange chromatography and designated as PUR20C, POR20C, PUR20A, and POR20A, respectively. The antibacterial activity of PUR20C, POR20C, PUR20A, and POR20A is shown in Table 2. The peptides purified by cation exchange chromatography showed increased antibacterial activity against all three bacteria, with the peptide derived from the jellyfish umbrella segment demonstrating greater antibacterial potency than that from the jellyfish oral arms segment. Conversely, peptides purified through anion exchange chromatography displayed diminished antibacterial activity across all three types of bacteria. Cationic peptides show superior antibacterial activity to anionic peptides due to their capacity to interact with negatively charged bacterial membranes electrostatically. This interaction facilitates membrane disruption, ultimately leading to cell lysis [28], [31], [32].

Table 2: The antibacterial activity of jellyfish peptide after purification by cation or anion exchange chromatography and reverse-phase chromatography.

Samples		Antibacterial Activity (%inhibition)		
		*E. coli**	*V. parahaemolyticus**	*S. aureus**
Cation	PUR	24.53±0.90^{A}	25.73±0.35^{A}	21.13±0.49^{A}
	POR	16.05±0.52^{B}	15.24±0.50^{B}	16.11±0.51^{B}
Anion	PUR	2.73±0.59^{C}	11.44±0.51^{C}	4.90±0.76^{C}
	POR	0.17±0.09^{D}	3.13±0.70^{D}	1.34±0.67^{D}

*Within each column, values marked with different superscript letters (A-D) reflect significant variation at the p-value < 0.05 level.

In contrast, anionic peptides necessitate specific cofactors—such as divalent metal ions—to exert their antibacterial effects [33]. Previous research corroborates these findings, as Park *et al*., [34] reported that peptides modified with lysine (K) exhibit enhanced antibacterial activity. The incorporation of

lysine increases the cationic nature of the peptide, strengthening its interactions with the negatively charged surfaces of target bacterial cells. Additionally, Stark *et al*., [28] noted that the cationic properties of peptides enhance antimicrobial activity by promoting cell membrane insertion. Thus, cationic peptides are of significant interest to research in discovering and developing new antimicrobial agents. Given its highest antibacterial activity, the PUR20C sample was selected for peptide sequencing using LC-MS/MS in conjunction with Mascot software.

Table 3: The peptide sequences and properties.

Peptide No.	Peptide Sequence	Hydrophobic Ratio	Charge	pI	Molecular Weight (Da)	Secondary Structure
1	NQKAMQELNE	30	–1	4.15	1204.32	hhhhhhhhhh
2	TDSPAPSETTD	9	–3	0.69	1120.09	ccccccceee
3	EQIYPMGEGDEL	25	–4	0.53	1380.49	ceeccccccch
4	PFTMYFLL	63	0	3.81	1031.28	chhhhhhh
5	PMETDDQPNN	10	–3	0.59	1160.18	ccccccccc

*pI = isoelectric point, h = α-helix, c = random coil, e = β-sheet.

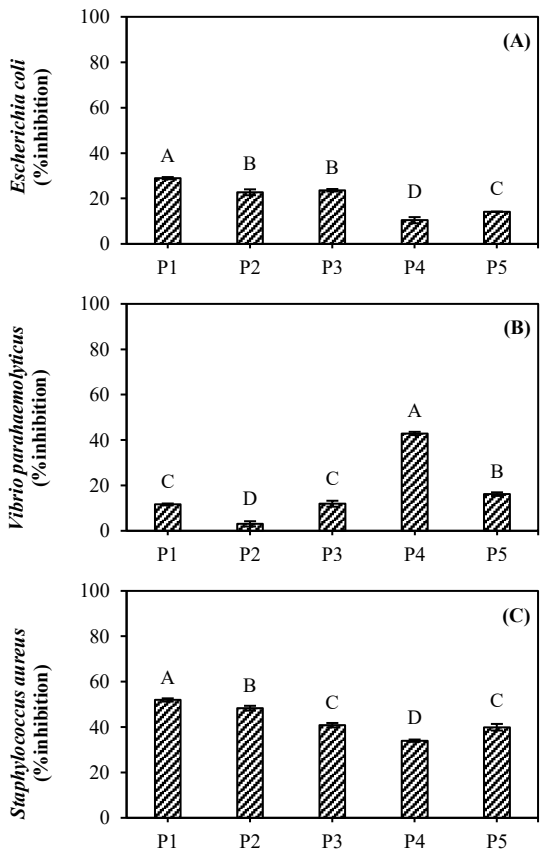

Figure 2: Antibacterial activity; (A) inhibitory percentages against *E. coli*, (B) inhibitory percentages against *V. parahaemolyticus*, and (C) inhibitory percentages against *S. aureus* of synthetic peptides. P1–P5 = Peptide No.1–5. Bars labeled with different letters within the same bacteria exhibit statistically significant differences (p-value < 0.05).

3.3 *Antibacterial activity of synthetic peptides*

In this study, we analyzed and sequenced thousands of peptides using LC-MS, ultimately selecting five peptides with high peptide scores, each smaller than 1500 Da, for synthesis (Table 3). The antibacterial activity of these five synthetic peptides is illustrated in Figure 2.

The results demonstrate that the peptides exhibited inhibitory activity against *E. coli,* ranging from 10.43% to 28.95%, against *V. parahaemolyticus* from 3.05% to 42.88%, and against *S. aureus* from 33.99% to 51.93%. Peptide No. 1 (NQKAMQELNE) displayed the highest inhibitory effects against *E. coli* and *S. aureus*, with values of 28.95% and 51.93%, respectively. In contrast, peptide No. 4 (PFTMYFLL) showed the most significant inhibitory activity against *V. parahaemolyticus* at 42.88%.

Various characteristics, including hydrophobicity, charge, isoelectric point, molecular weight, and structure, influence these peptides' antibacterial efficacy [28], [30], [31]. Interestingly, some of these synthetic peptides demonstrated higher antibacterial activity compared to the peptide fractions purified by cation exchange chromatography. One possible explanation is that synthetic peptides are designed with known amino acid sequences, allowing precise control over key structural features associated with antibacterial potency, such as net positive charge, amphipathicity, and optimal hydrophobicity [30], [31], [35]. In contrast, the peptide mixtures obtained from cation exchange chromatography consist of heterogeneous peptides of unknown sequences and varying characteristics. This complexity may dilute the bioactivity of individual peptides with strong

antibacterial potential, resulting in overall lower activity. The superior activity of the synthetic peptides observed in this study underscores the importance of peptide sequence specificity in determining antimicrobial effectiveness. This study found that negatively charged peptides enhanced inhibitory activity against *E. coli* and *S. aureus*, while positively charged peptides were more effective against *V. parahaemolyticus*.

Furthermore, peptides No. 1 and No. 4 exhibited vigorous antibacterial action due to their high hydrophobicity, elevated isoelectric points, and predominantly α-helix (h) structure. α-helix are often linked to potent antibacterial activity because of their amphipathic nature, featuring one hydrophobic side and one hydrophilic or charged side. This unique configuration allows them to insert into and disrupt bacterial membranes [35], [36]. The 3D structures of the five synthesized peptides are presented in Figure 3, revealing that peptides with a helical structure possess greater antibacterial activity than those with a β-sheet (e) or random coil (c) structure.

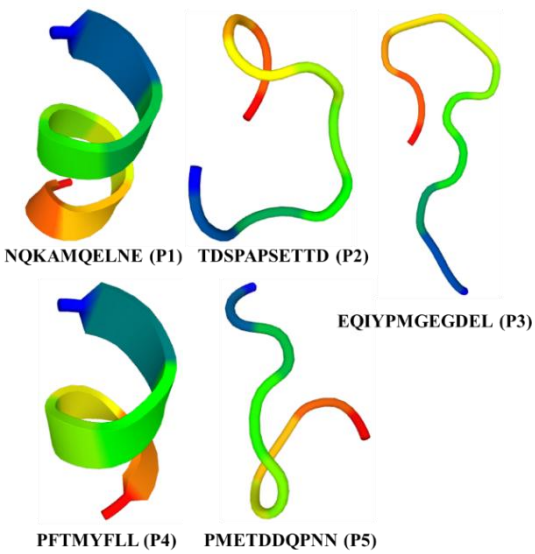

Figure 3: 3D structure of synthesized peptides obtained using the PEP-FOLD3 tool. The color gradient in the peptide 3D structure represents the directionality of the amino acid sequence, with colors transitioning from red to blue to indicate the N-terminal to C-terminal progression.

3.4 *Anti-α-amylase activity of synthetic peptides*

α-Amylase is an enzyme essential for the digestion of starch and glycogen, which are complex carbohydrates (polysaccharides) [15]. It functions by hydrolyzing α-1,4-glycosidic bonds in starch or glycogen, thereby breaking these substances down into smaller units such as maltose, maltooligosaccharides, and glucose, which can then be absorbed into the bloodstream [15], [16]. Consequently, inhibiting the α-amylase enzyme can help reduce glucose absorption into the blood [37]. and ionic bonds, enhancing the stability of enzyme binding and resulting in a strong inhibitory effect of the peptide [38]. The anti-α-amylase activity of five synthetic peptides is illustrated in Figure 4. All peptides demonstrated anti-α-amylase activity, with peptide No. 1 (NQKAMQELNE) exhibiting the highest inhibitory effect, achieving 100.00% at a 100 µg/mL concentration. This inhibitory percentage is notably higher than that reported by Zhou *et. al.,* [39], who found that the peptide MMFPH exhibited 66.41% inhibition at the same concentration of 100 µg/mL. Furthermore, it also exceeds the inhibition reported by Zhong *et al*. [40], where oyster-derived peptides demonstrated anti-α-amylase activity of 64.68% at a higher concentration of 300 µg/mL. These comparisons suggest that the peptide NQKAMQELNE possesses a remarkably strong inhibitory effect against α-amylase, even at lower concentrations. The presence of polar amino acids, particularly lysine (K) and glutamic acid (E), in the peptide chain is crucial for the formation of hydrogen and ionic bonds, enhancing the stability of enzyme binding and resulting in a strong inhibitory effect of the peptide [38].

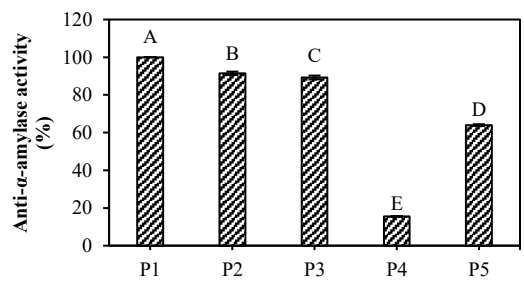

Figure 4: Anti-α-amylase activity of synthetic peptides. Distinct letter labels atop the bars signify statistically meaningful differences (p-value < 0.05).

3.5 *Anti-α-glucosidase activity of synthetic peptides*

α-Glucosidase is an enzyme that hydrolyzes carbohydrates by cleaving the α-1,4-glycosidic bonds found in disaccharides and oligosaccharides, such as maltose and maltooligosaccharides, to release glucose [16], [41]. This process aids in the absorption of glucose into the body. Inhibiting the α-glucosidase

enzyme can delay glucose absorption into the bloodstream, thus lowering the intensity of postprandial blood sugar spikes [42]. Consequently, peptides exhibiting α-glucosidase inhibitory activity hold potential as treatments for type 2 diabetes or as nutraceuticals in health foods. The anti-α-glucosidase activity of five synthesized peptides, illustrated in Figure 5, revealed that their inhibitory effects ranged from 16.27% to 70.48%. Previous studies have indicated that the presence of proline (P) and tyrosine (Y) residues in the peptide chain can significantly enhance their inhibitory potency against α-glucosidase [43], [44].

Additionally, the secondary structure of peptides, particularly random coils, is crucial for effective enzyme inhibition due to their flexibility and adaptability, allowing them to fit into the enzyme's binding site more efficiently than their rigid counterparts. These flexible conformations can mimic the structure of the natural substrate, thereby facilitating enzyme binding and inhibiting its activity [45], [46]. As a result, peptide No. 3 (EQIYPMGEGDEL), which contains both proline and tyrosine and predominantly adopts a random coil secondary structure, demonstrates the highest anti-α-glucosidase activity.

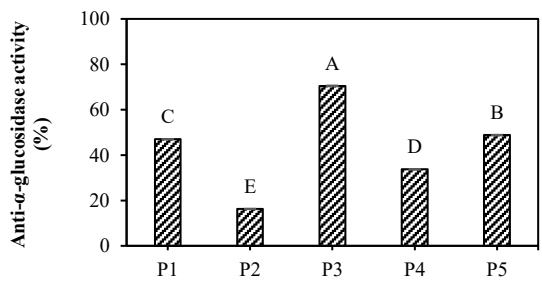

Figure 5: Anti-α-glucosidase activity of synthetic peptides. Statistically significant differences (p-value < 0.05) are indicated by different letters above the bars.

3.6 *Hemolytic activity of synthetic peptides*

The hemolytic activity of the synthesized peptides was assessed to evaluate their potential cytotoxicity toward mammalian cells. As illustrated in Figure 6, incubation of red blood cells with the five synthetic peptides (100 μg/ml) resulted in minimal hemolysis, with lysis rates ranging from 4.14% to 7.12%. Generally, peptides exhibiting hemolytic activity below 10% at working concentrations are classified as having low toxicity and are deemed suitable for further biological applications [47]. Consequently, all peptides investigated in this study displayed hemolytic activity below the 10% threshold, indicating they possess very low cytotoxicity and can be considered non-toxic to mammalian cells.

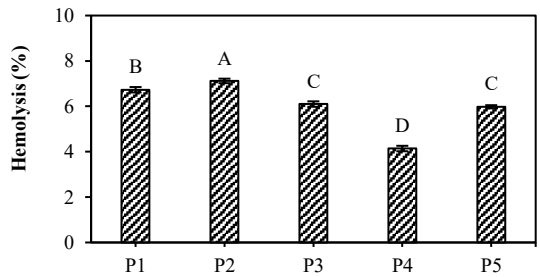

Figure 6: Hemolytic activity of synthetic peptides. Bars sharing no common letters are significantly different (p-value < 0.05).

3.7 *Antibacterial mechanisms of synthetic peptides*

The logical relationships between expressed proteins in both control and treated samples across different treatments were illustrated using a Venn diagram (Figure 7). In *E. coli* treated with peptide No. 1, a total of 268 expressed proteins were identified. Of these, 92 proteins were uniquely expressed in the sample treated with peptide No. 1, while 24 were also observed under kanamycin treatment. In the case of *V. parahaemolyticus* treated with peptide No. 4, 21 expressed proteins were identified, with 11 being unique to peptide No. 4 treatment. For *S. aureus* treated with peptide No. 1, 59 expressed proteins were detected, of with 50 were unique to this treatment.

The protein expression Venn diagrams (Figure 7) reveal that the majority of proteins expressed by pathogenic bacteria in response to synthetic peptides were unique, and these proteins were not observed in samples treated with antibiotics. Indicating that the mechanisms of action for peptide No.1 and peptide No.4 differ from those of antibiotics. Further insights into these mechanisms have been derived using UniProt software.

Initially, we categorized all proteins expressed in samples treated with peptide (which were not expressed in the antibiotic treatment or control) according to their Gene Ontology annotations (Figure 8). In the case of *E. coli* treated with peptide No. 1, a significant portion of the expressed proteins was associated with DNA transposition (17.64%), alongside others related to biological processes involving DNA, including DNA repair, transcription,

transposition, recombination, restriction-modification systems, methylation, integration, and replication. These findings indicate that the antibacterial mechanism of peptide No. 1 is linked to intracellular processes.

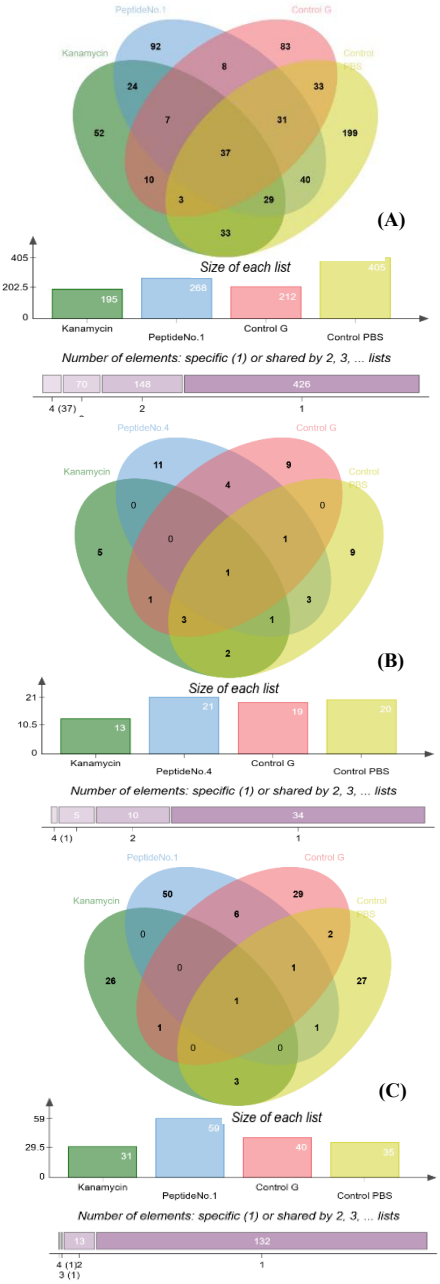

Figure 7: Venn diagram summary of proteins identified in pathogenic bacteria treated with synthetic peptides and antibiotics: (A) *E. coli*, (B) *V. parahaemolyticus*, and (C) *S. aureus*. The number of elements indicates the number of proteins detected in each treatment.

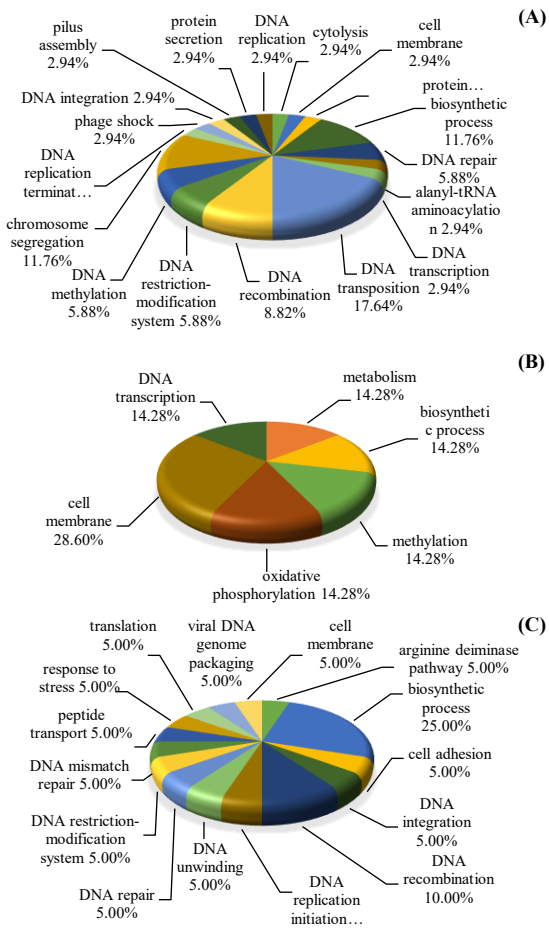

Figure 8: Functional profiles of identified proteins expressed in the samples treated with peptide (but not expressed in antibiotic treatment or control); (A) *E. coli* treated with peptide No. 1, (B) *V. parahaemolyticus* treated with peptide No. 4, and (C) *S. aureus* treated with peptide No. 1.

In *V. parahaemolyticus* treated with peptide No. 4, the majority of expressed proteins were associated with the cell membrane (28.60%). However, over 50% of the other expressed proteins were involved in functions related to biosynthetic processes, metabolism, methylation, oxidative phosphorylation, and DNA transcription. As a result, it is likely that these peptides induce cell leakage, with some also influencing intracellular processes.

Lastly, we analyzed the functions of proteins expressed in *S. aureus* treated with peptide No. 1. The majority of expressed proteins (25%) were associated

with biosynthetic processes. Other functions included various biological processes involving DNA, such as integration, recombination, replication initiation, unwinding, repair, restriction-modification systems, and mismatch repair. Furthermore, a few proteins were linked to the cell membrane. Therefore, peptide No. 1 can be characterized as an intracellular-active antibacterial peptide.

Following an examination of peptide No. 1's mechanism against *E. coli* and *S. aureus*, it was determined to possess characteristics of an intracellularly active antibacterial peptide. Such peptides can inhibit or eliminate microbial cells without disrupting their membranes. Instead, they interact with various substances within the cell, including proteins, DNA, or RNA [23], [48], [49]. Likewise, peptide No. 4, when tested on *V. parahaemolyticus*, was also identified as an intracellularly active antibacterial peptide. Notably, while peptides No. 1 and No.4 primarily exhibit intracellular activity, they also influence the expression of certain proteins associated with the cell membrane, particularly in the case of peptide No. 4. These findings align with previous research findings. Ditsawanon *et al*., [23] noted that peptides derived from agricultural waste have dual mechanisms for inhibiting microorganisms: one targeting the cell membrane (which includes cell wall organization and integral membrane components), and the other acting within the cell (affecting proteins, nucleic acids such as DNA and RNA, and lipids).

4 Conclusions

This study successfully highlighted the potential of pepsin-derived peptides from low-cost salted jellyfish byproducts as multifunctional bioactive compounds. The purified peptides were obtained through reverse-phase and ion exchange chromatography, demonstrating enhanced antibacterial activity compared to their crude hydrolysate counterparts. This underscores the significance of purification in enhancing bioactivity. The synthesized peptides exhibited notable antibacterial effects against key pathogenic bacteria and significant inhibitory effects on α-amylase and α-glucosidase while maintaining minimal hemolytic activity, indicating their safety for potential applications. NQKAMQELNE emerged as particularly noteworthy among these peptides due to its strong bioactivities and low cytotoxicity, with its antimicrobial mechanism linked to interference with intracellular processes. The findings suggest that jellyfish byproducts, often viewed as waste, can be converted into valuable functional ingredients, promoting environmental sustainability and offering natural alternatives for the food and pharmaceutical industries. In addition to bioactive peptides, these by-products could also serve as a source of other valuable compounds such as collagen, gelatin, or antioxidant peptides, which hold promising applications in cosmetics, nutraceuticals, and biomaterials. Moreover, the residual protein fractions could potentially be repurposed for agricultural uses, such as organic fertilizers or microbial growth media. These prospects further emphasize the comprehensive utility of jellyfish by-products and support their valorization in various industrial sectors. However, further research into the mechanisms of these peptides and their *in vivo* efficacy is essential to realize their potential applications fully.

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Author Contributions

P.M.: conceptualization, investigation, data analysis, methodology, formal analysis, research design, data curation, visualization, validation, writing an original draft, and writing-reviewing and editing; B.T. and S.R.: conceptualization, methodology, formal analysis, visualization, validation, writing-reviewing and editing, project administration, funding acquisition, and supervision; W.C.: methodology, and visualization; S.C. and B.W.: methodology; V.R. and F.C.: validation, and writing-reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

The MS/MS raw data and analysis are available in the ProteomeXchange Consortium via the jPOST partner repository: JPST003792 and PXD063639.

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