Antibacterial activity of protein of marine fish *Sphyraena putnamae* (Jordan and Seale, 1905) of Gulf of Mannar, south eastern India

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Abstract

Fish is an important and cheap source of essential nutrients. Protein of *Sphyraena putnamae* was extracted and the crude fish protein was partially purified by dialysis and quantified by Bradford analysis against the BSA protein standard. The concentration of the partially purified protein of *S. putnamae* was estimated to be 2.00±0.75 mg/g. The antimicrobial activity of the fish protein was studied by agar well diffusion method. The protein sample at different concentrations inhibits bacterial growth and the study recorded the zone of inhibition against *Streptococcus oralis* and *Streptococcus pyogenes*. According to the results, the protein sample exhibits concentration-dependent inhibition against bacterial species. The maximum inhibition was observed at 500µg/ml and the zone of inhibition was 15.00 ± 2.00 mm against *Streptococcus pyogenes* and 11.25 ± 0.35 mm against *Streptococcus oralis*. This study demonstrates that the protein of *Sphyraena putnamae* possessing antibacterial activity can be extracted, identified and further developed into new pharmacological products.

INTRODUCTION

Marine environment provides excellent bio-resources that have been identified, processed and utilized for human welfare. Some of the potential bioactive molecules are antimicrobial peptides (AMP), which are small proteins present in various plants and animals. They act as the first line of defense against microbes. The AMPs secreted by marine organisms confer them immunity against a variety of microorganisms of the aquatic environment. The resistance developed by pathogens against conventional antibiotics and the need for novel antibiotics have stimulated interest in the development of antimicrobial peptides for human use (Zasloff, 2002). Fish is considered the main source of proteins worldwide; furthermore, fish proteins have an enormous potential as novel sources of bioactive peptides that can kill pathogenic microbes (Yathisha *et al.,* 2019). The hydrolysates of fish proteins have been found to exhibit several biological actions such as anti-inflammatory, antihypertensive, antioxidant, antimicrobial, immunomodulatory and antithrombotic activities, opioid action and anti-proliferative effects apart from their role in mineral absorption (Phelan et al., 2009; Najafian and Babji, 2012; Cheung *et al.,* 2015; Kang *et al.,* 2019). Among the countless biological activities already studied in fish muscles, antimicrobial activity of fish peptides is important and it

represents the ancient host-defense effector molecules (Patel and Akhtar, 2017). Recently, antimicrobial peptides have been attracting the attention of researchers as possible substitutes for conventional antibiotics, because microbes often develop resistance to those antibiotics, which has become a great challenge in the treatment of diseases (Aoki and Ueda, 2013). Antimicrobial peptides possess amphipathic properties due to their positive charges and hydrophobic residues, and contribute to the innate immunity of the fish against pathogenic organisms. The mechanisms of the actions that antimicrobial peptides exhibit are remarkably different from those of commonly used antibiotics, as they are able to lyse bacterial membranes and inhibit cellular metabolic processes including the biosynthesis of DNA and protein, protein folding and metabolic turnover (Brogden, 2005;). AMP shows high antimicrobial activity even in very low concentrations against a broad spectrum of microorganisms, including gram-positive and gram-negative bacteria, fungi, viruses and pathogens specific to fish (Brogden, 2005). The isolation, identification and characterization of AMPs are therefore of great importance for possible use in the pharmaceutical and nutraceutical sectors.

Many studies have already demonstrated that a broad spectrum of AMPs are found in fish proteins including defensins, cathelicidins, hepcidins, mytilin, histone-derived peptides, and the fish-specific piscidins, which belong to the cecropin family (Masso-Silva *et al.,* 2014). It is widely recognized that AMPs are encrypted in parent proteins and they are in their inactive form and activated by protein digestion (Valero *et al.,* 2020). Antimicrobial peptides generally possess a minimum of 12 and a maximum of 50 amino acids in their sequences (Mohanty *et al.,* 2016) and generally have cationic, amphipathic and α-helical characteristics, though some non-cationic antimicrobial peptides have also been reported (Lakshmaiah and Chen, 2015). Proteins of fish are essential food constituents with nutritional and physiological properties. They are rich in essential amino acids, which are required for the growth and development of the body. Bioactive peptides are inert in the original protein chains but can be activated by proteolytic digestion when needed. Bioactive peptides such as AMPs have received increasing attention and been recommended as prophylactic and therapeutic substances for a variety of ailments (Marqus *et al.,* 2017). Food-derived antibacterial peptides can serve as beneficial alternatives to harmful chemical preservatives and can be safely used in food preservation (Przybylski *et al.,* 2016). However, proteins require purification and characterization before being marketed and used. Dialysis is a technique usually employed in the purification of proteins that facilitates the removal of small, unwanted compounds from macromolecules in solution by selective and passive diffusion through a semi-permeable membrane. SDS-PAGE is the most commonly used gel electrophoretic system for separation of proteins. This method is based on the separation of proteins according to size and can also be used to determine the approximate molecular mass of proteins. SDS is an anionic detergent which binds strongly to protein backbone and denatures proteins to produce linear polypeptide

chains eliminating the influence of structure and charge.

Nowadays resistance developed by bacterial and fungal pathogens against antibiotics is one of the major problems faced in all countries (Arias and Murray, 2009). It has resulted in the increasing demand for newer antimicrobial therapies that suppress this resistance. Marine animals are potent source of active molecules with pharmacological significance. Thousands of bioactive compounds have been discovered in marine algae, sponges, microbes and seafood such as finfish, crustaceans and mollusks. They are peptides, sterols, terpenes, polypropinate, nitrogenous compounds, fatty acid derivatives, miscellaneous compounds and alkaloids (Blunt *et al.,* 2009). Many elements of its qualities have been elucidated in recent years by numerous researches on fish muscle composition, but there is still more to learn about its antibacterial activity. However, in India there has been no thorough report on the antibacterial properties of fish proteins. It is important to assess the antibacterial and antifungal properties of proteins obtained from animal flesh in light of the recent studies on the development of bioactive peptides generated from marine protein as an alternative to manufactured medications for the treatment of various ailments. The objective of this work is to extract proteins from an economically significant fish *S. putnamae*, and assess their antibacterial activity against pathogenic bacteria to identify the possible biomedical applications.

MATERIALS AND METHODS

Sample collection

Healthy individuals of *Sphyraena* sp. weighing 500-600 g were collected from the fish landing centre of Therespuram fishing village, Tuticorin, Tamil Nadu and carefully brought to the laboratory in an ice box for protein extraction.

Fish sample preparation

The fish samples were washed with distilled water, the fish scales scraped gently off and the skins removed. Then the samples were washed three times with 0.8% NaCl. All the extraction procedures were carried out at 4° C to avoid protein denaturation and stored at -20° C prior to use.

Extraction of fish protein using Tris-HCL Method

The tissue samples (100 g) were homogenized with 10 mM Tris-HCL buffer (pH-7) containing a mixture of protease inhibitors on ice for 2 min using homogenizer. The homogenate was centrifuged at 15,000 rpm g for 30 min at 4° C. The pellet was discarded and the supernatant containing soluble protein was collected and stored in -80° C until use. Protein sample of 50µl was mixed with 2:1 ratio of sample buffer (Tris–HCl, pH 6.8 containing 2-mercaptoethanol, glycerol, bromophenol blue, 5 % SDS) and heated at 90° C for 5 min.

Partial purification of protein by dialysis method

Dialysis membrane was placed in 0.3% NaCl (80° C) for 1 min., then washed with hot distilled water (60° C) for 2 min and kept in 0.2 N sulfuric acid for 1 min. Then 10 ml of sample was loaded

into the membrane and kept in 0.5 M phosphate buffer (4° C) overnight. After the sample was collected, 10% SDS PAGE analysis was carried out.

Separation of fish protein by SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) to determine the molecular weight of *S. putnamae* proteins. Then, 25 µl of sample was loaded along with high molecular weight protein markers. Electrophoresis was carried out at 50 mA initially and then at 100 mA. Protein bands were stained with Coomassie Brilliant Blue R250. The molecular weights were determined by comparison with standard protein markers. The samples were mixed with 2:1 ratio of sample buffer and heated up to 90° C for 3 min. Then they were cooled immediately. The samples were loaded in the gel and run in 50 V till the samples crossed the stacking gel. Then current was raised to 100 V. The electric field was turned off after 10 min of dye run out.

Coomassie brilliant blue staining

Following electrophoresis, the gel was placed in the Coomassie staining solution and incubated for 6 hours. Then the gel was kept in a de-staining solution until the background was transparent and the bands were observed.

Quantification of fish protein using Bradford Analysis Method

The partially purified fish proteins were quantified using the standard Bradford method. Bradford's reagent binds to the amino acids of the unknown (fish) protein solution, resulting in a colour change from brown to darker shades of blue. The absorbance was read at 595 nm, which is proportional to the amount of protein bound. Bovine Serum Albumin was used as the standard. The amount of protein in the fish sample was calculated from the standard graph.

Antibacterial Activity

Bacterial cultures

Two bacterial strains namely *Streptococcus oralis* (MTCC 2696) and *Streptococcus pyogenes* (MTCC 1928) were used to demonstrate the antibacterial activity of the partially purified protein of *S. putnamae*. The strain *Streptococcus oralis* is an opportunistic pathogen which can enter the blood stream causing bacteremia and infective endocarditis (Chamat-Hedemand et al 2020). The other strain *Streptococcus pyogenes* is a human pathogen causing mild to invasive infections like necrosis and toxic shock syndrome (Ibrahim et.al 2016).

Antibacterial activity

Agar well diffusion method

Nutrient broth was prepared by dissolving 2.8 g of commercially available nutrient broth medium (HiMedia) in 100 ml of distilled water and boiled to dissolve the medium completely. The medium was sterilized by autoclaving at 150 lbs pressure (121° C) for 15 min. Petri plates containing 20 ml nutrient agar medium were seeded with 24-hour culture of the two bacterial strains namely *Streptococcus oralis* (MTCC 2696) and *Streptococcus pyogenes* (MTCC 1928). Briefly, nutrient agar plates were inoculated with bacterial strain under aseptic conditions and the wells (diameter = 6 mm) were filled with

test samples (of 500, 250, 100 and 50 µg/ml) and incubated at 37° C for 24 hours. After the incubation period, the diameter of the growth inhibition zones was measured. Single colonies cultured for 18 to 24 hours on agar plates were used to prepare the bacterial suspension with the turbidity of 0.5 McFarland (equal to 1.5×10^8 colony-forming units (CFU/ml). Turbidity of the bacterial suspension was measured at 600 nm. Gentamicin antibiotic was used as a positive control. The values were calculated using GraphPad Prism 6.0 software (USA).

Statistical analysis

GraphPad Prism 6.0 software package with three times in triplicates for LC50 value calculation (n = 50). All the other experiments were performed in triplicates and results are presented as mean ± standard deviation. One way analysis of variance (ANOVA) was carried out for statistical analysis and the significance level was set as $P < 0.05$, $P < 0.01$ and $P < 0.001$. Further, mean values were compared by post-hoc Tukey's test. The graphical representations were performed using GraphPad Prism Software.

RESULTS

The crude protein of fish *S. putnamae* (5 ml) was partially purified using the protein dialysis method . The partial purification of fish protein was done against 0.5 M phosphate buffer overnight at 4° C.

Separation of partially purified fish protein by SDS PAGE method

After the incubation, the dialyzed protein sample was collected and resolved in 10% SDS PAGE. The partially purified protein sizes were found to be below 45 kDa,
30 kDa, 25 kDa, 18 kDa and 14 kDa respectively. The five bands are shown in Fig 1. The 25 kDa proteins show higher intensity than the other protein bands.

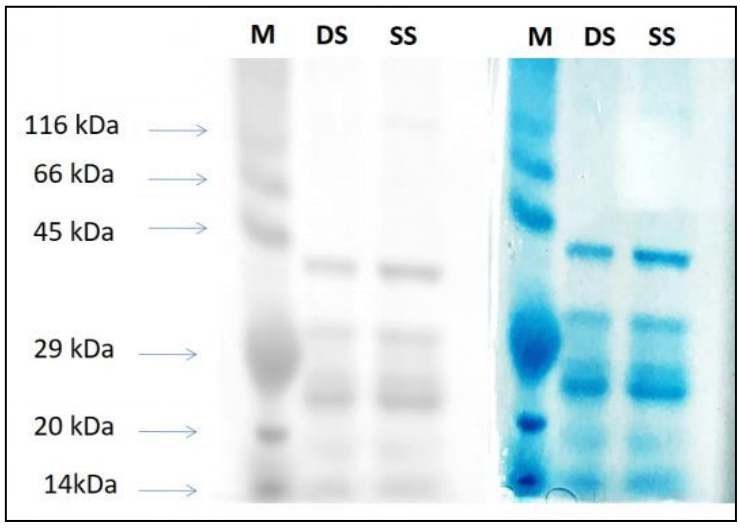

Fig 1 SDS-PAGE (10%) showing the partially purified fish protein by dialysis; L - Protein Ladder, DS – Dialyzed Sample, SS – Sample in sodium phosphate buffer

Quantification of partially purified protein by Bradford analysis

The partially purified proteins were further quantified using Bradford analysis against the BSA protein standard and the OD value observed at 595 nm. The unknown protein concentration of the partially purified *S. putnamae* protein sample was quantified as 2.00±0.75 mg/g.

Antibacterial activity of partially purified fish protein

The antibacterial activity of the partially purified fish protein was tested against two different bacterial pathogens. The results show that the protein sample has concentration-dependent inhibition against bacterial species. The maximum inhibition was observed at 500 µg/ml, with the zone of inhibition observed at 15.00±2.00 mm against *Streptococcus pyogenes* and 11.25±0.35 mm against *Streptococcus oralis*. The results are presented in Fig 2 and Fig 3 and Table 1.

Antibacterial activity of partially purified fish extract against bacterial pathogens

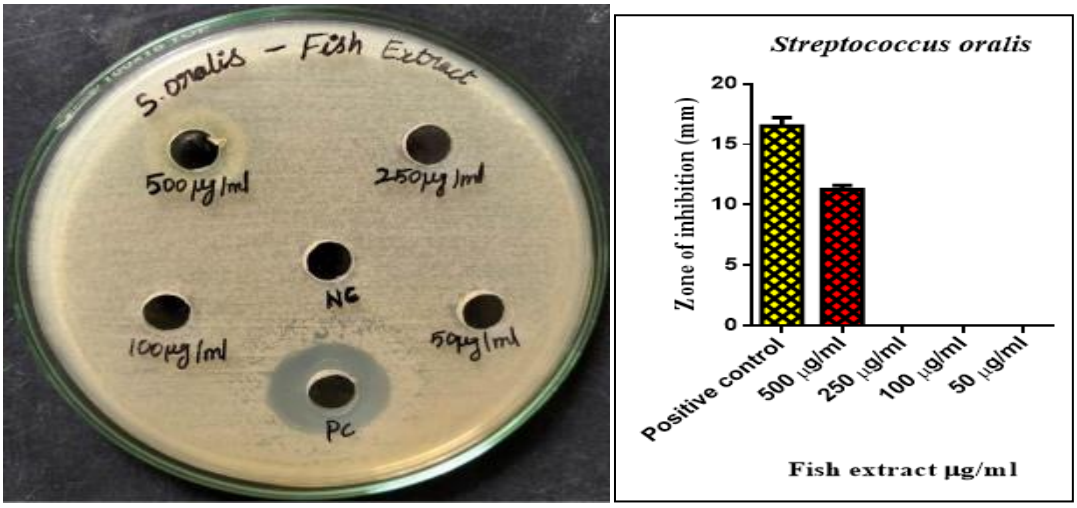

Fig 2 Effect of sample fish extract against *Streptococcus oralis*

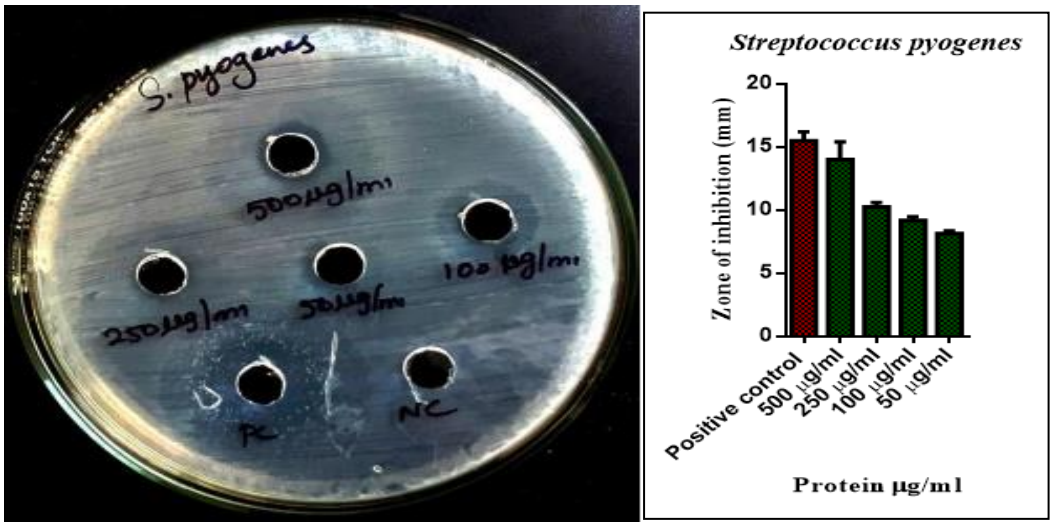

Fig 3 Effect of sample fish extract against *Streptococcus pyogenes*

Table 1 SD ± Means of zone of inhibition obtained by sample fish extract against *bacterial species*

S No	Name of the test organism	Name of the test sample	Zone of inhibition (mm) SD ± Mean				
			500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	PC
1.	*Streptococcus oralis*	Fish protein extract	11.25±0.35	0	0	0	16.5±0.7
2.	*Streptococcus pyogenes*		15±2.0	10.5±0.5	9.4±0.4	8.3±0.3	16±1.0

SD – Standard Deviation, *Significance - $p< 0.05$

DISCUSSION

A substance used to kill or suppress the growth of microorganisms is called an antimicrobial agent. Antimicrobial activity has previously been described in a wide range of fish species (Shike et al., 2002; Patrzykat and Aleksander, 2003; Douglas et al., 2003; Rodrigues et al., 2006; Sun et al., 2007; Kim &Wijesekara, 2010; Chen et al., 2010; Browne *et al.,* 2011; Broekman *et al.,* 2011; Buonocore et al., 2012). The present study demonstrates the antibacterial activity of the partially purified protein of fish *Sphyraena putnamae*. Research regarding the isolation and identification of antimicrobial peptides derived from animal muscle has not been as extensive as for antioxidant peptides from animal muscle. Fish are a major component of the aquatic fauna. Like other organisms, fish exude different types of antimicrobial peptides, which are positively charged short amino acid chain molecules involved in host defense mechanisms. Antimicrobial peptides play key roles in native immunity by interacting directly with bacteria and killing them. A wide variety of organisms produce antimicrobial peptides as a primary innate immune strategy. In medicinal applications, antimicrobial peptides are sometimes preferred to conventional bactericidal antibiotics because they kill bacteria faster and are unaffected by antibiotic-resistance mechanisms (Shahidi and Zhong, 2008). The methods described here will be useful for the identification of novel peptides with good antimicrobial activities. Several methods for testing the antimicrobial activity of hydrolysates or peptides have been used. The agar diffusion assay (or inhibition zone assay) is a common method used to test the antimicrobial activity of peptidic hydrolysates and peptides (Hickey *et al.,* 2003). This method quantifies the ability of antibiotics to inhibit bacterial growth. The agar diffusion technique is usually employed in determining the minimum inhibitory concentration (MIC) in solid media. Antibiotic diffusion from these sources

into the agar medium leads to inhibition of bacterial growth in the vicinity of the source and the formation of clear zones without a bacterial lawn. The diameter of these zones increases with antibiotic concentration. A novel polypeptide with antimicrobial activity was isolated and characterized from loach (*Misgurnus anguillicaudatus*) using Sephadex G-50 gel filtration, DEAE-52 cellulose ion-exchange chromatography and an improved polyacrylamide gel electrophoresis together with electroelution (Dong *et al.,* 2012). In the present study protein was partially purified by the dialysis method. Zhang *et al.* (2008) found an antimicrobial component in the skin homogenate of *Epinephelus fario* using a trypsin digest. The antimicrobial protein was purified by ion-exchange and gel-filtration chromatography. Najafian and Babji (2012) isolated proteinaceous antibiotic from the skin homogenate and purified it using a DEAE-Sephadex A-50 column. The purified protein, named Efap, had a molecular mass of 41 kDa.

Zhang *et al.* (2008) found fish protein to have higher antimicrobial activity against Gram-negative bacteria (*V. parahaemolyticus, V. alginolyticus, V. fluvialis, P. multocida* and *A. hydrophila*) than against Gram-positive bacteria (*S. aureus*). In this study *Streptococcus oralis* and *Streptococcus pyogenes* are the bacterial strains used and Gentamicin is the positive control of the test. The test sample concentration was taken as 50, 100, 250, and 500 µg/ml and loaded in the wells made in the agar after incubation. The results show that the zone of inhibition is higher for 500 µg/ml concentration than for the others. The zone of inhibition was found to be 11.25±0.35 and 15.00±2.00 at 500 µg/ml respectively for *Streptococcus oralis* and *Streptococcus pyogenes*. The positive control's zone of inhibition was found to be 16.50±0.70 and 16.00±1.00 for *Streptococcus oralis* and *Streptococcus pyogenes* respectively. The p-value of the test was set at $p<0.05$, which indicates the conclusions to be highly significant.

Deivasigamani *et al.* (2017) prepared the muscle tissue extract of marine finfish *Mugil cephalus* and evaluated its antibacterial activity against *E. coli, Proteus mirabilis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. A maximum zone of inhibition of 29 mm diameter was formed against *Proteus mirabilis* followed by *Staphylococcus aureus* with a zone of inhibition of 16 mm in diameter. The fish tissue extract was found to exhibit minimum antibacterial activity against *P. aeruginosa* with 4.14 mm zone of inhibition. Anil *et al.* (2016) studied the antibacterial properties of protein extract of fresh water fish *Hypophthalmichthys nobilis* against some human and fish pathogens such as *Klebsiella pneumonia, Staphylococcus epidermis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus cereus* and *Aeromonas hydrophila*. The aqueous mucus extract produced zone of inhibition at 13.16±0.49, 16.71±1.04, 12.73±0.51, 16.55±1.10, 11.58±0.50, 15.85±0.94 and 16.03±1.16. In the present study the 500 µg/ml fish protein extract was found to exhibit minimum antibacterial activity against *Streptococcus pyogenes* with 15 mm zone of inhibition. Antimicrobial peptides of marine organisms are the existing candidates for the development of new

antibacterial compounds thanks to their broad activity spectrum and difficulty for bacteria to develop resistance to them.

In another study Mehrnoosh *et al.* (2020) detected the antibacterial activities of the extracts of various organs of sea cucumber *Holothuria leucospilota.* Antibacterial activity was strongly exhibited by body wall and gonad extracts against Gram-positive bacteria *Streptococcus aureus* and moderate activity against other Gram-positive and Gram-negative bacteria. Deepak *et al.* (2020) evaluated the antimicrobial potential of marine gastropods *Paphia malabarica* and *Crassostrea gryphoides* against six bacterial strains *Pseudomonas aeruginosa, Corynebacterium diphtheria, Salmonella paratyphi B, Staphylococcus aureus, Proteus vulgaris* and *Escherichia coli* and the fungi strains *Malassezia furfur* and *Aspergillus niger.* Metronomic extracts of the bivalve *P. malabarica* showed 6 mm zone of inhibition for *Proteus vulgaris* and 4 mm for *Malassezia furfur*. Metabolic extracts of the oyster *Crassostrea gryphoides* showed 4 mm zone of inhibition for *C. diphtheria* and 4 mm for *Aspergillus niger*. Srikanya *et al.* (2018) demonstrated the antimicrobial activity of fish protein sample against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative strains such as *Escherichia coli.* The zone of inhibition increased as the concentration of the fish protein increased. A concentration of 1,000 ppm of fish protein hydrolysate produced 14.83±0.76 mm of zone of inhibition for *S. aureus,* 12.83±1.04 for *B. subtilis* and 11.17±1.04 for *E. coli.*

Hiwarale *et al.* (2016) evaluated the antibacterial activity spectrum of fish protein. The protein samples inhibited the growth of bacteria such as *Staphylococcus aureus, Corynebacterium* spp., *Streptococcus agalaciteae, Bacillus subtilis* and *Aeromonas hydrophilic.* Samaneh (2019) studied the antibacterial activity of protein hydrolysate of yellow-fin tuna *Thunnus albacores* and found that the lowest molecular weight fraction of less than 3 kDa showed the highest percentage of inhibition of bacterial growth against Gram-positive *Listeria sp.* and *Staphylococcus sp.* and Gram-negative *E. coli* and *Pseudomonas sp.* Baco *et al.* (2021) studied the antibacterial activity of functional bioactive peptides derived from fish protein hydrolysate consisting of 12 amino acids. It was found to be active against *Bacillus subtilis.* The inhibition zone increased with peptide concentration and the peptides were found to be active against Gram-positive and Gram-negative bacteria. Maryam *et al.* (2021) investigated the antibacterial effect of peptide fractions obtained from the skin collagen of fish *Huso huso*. The hydrolysate produced with papain and neutrase showed the most potent inhibitory effect against Gram-negative *Salmonella* strains including *S. abony, S. typhimurium* and *S. chol*. Yedery and Reddy (2009) studied the antibacterial activity of protein isolated from *Scylla serrate* against Gram-positive and Gram-negative bacteria. They found that the Gram-negative bacteria (*E. coli* and *P. aeruginosa*) to be more susceptible than Gram-positive ones tested (*S. aureus* and *S. pyogenes*). The results demonstrate that fish proteins are wonderful sources of antimicrobial agents with a wide range of antimicrobial properties. The results of the present study indicate that the antibacterial

factors are also produced in fish proteins. The antibacterial activity might be due to factors of the innate immune system. So, the animal protein can be used as an antimicrobial agent against many different pathogens. Recent studies have shown that fish-derived bioactive peptides play a vital role in human health and nutrition. More studies should be conducted to further explore the physiological effects of these peptides in humans. Fish-derived bioactive peptides provide a new source for the development of novel antimicrobial drugs in the future. Future vaccines using these antimicrobial peptides to neutralize particular diseases could potentially be employed as food preservatives and dietary supplements. The increase of dangerous microorganisms that are resistant to traditional antibiotics makes it crucial to discover new antimicrobial compounds. Fish-derived bioactive peptides have the potential to be exploited as active components in pharmaceuticals, food supplements, and functional foods as there is data supporting their positive benefits on health. We need to develop suitable techniques for the extraction, purification and identification of these bioactive peptides to enable their widespread application.

Conclusion

In this study, fish protein from *Sphyraena putnamae* demonstrated significant antibacterial action against Gram-positive and Gram-negative bacteria by disrupting the cell membrane integrity. Short-chain proteins with lower molecular weight have diverse, active chemicals that contribute to their antibacterial activity by forming an inhibitory zone. This knowledge can be used to optimize production conditions, increase the yield of selected protein, and gain a better understanding of the beneficial bioactive proteins involved in the development of antibacterial activity in a diverse selection of fish species.

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