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Review

Extraction of Novel Bioactive Peptides from Fish Protein Hydrolysates by Enzymatic Reactions

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Abstract: Bioactive peptides derived from fish the byproduct protein hydrolysate have wide potential as functional food ingredients. The preparation of bioactive peptides is commonly achieved via enzymatic hydrolysis; this is the most preferred method because it has high specificity, fewer residual organic solvents in the product, and it is usually carried out in mild conditions. The use of various enzymes such as proteases is widely practiced in the industry, yet there are various limitations as it is of high cost and there is a limited availability of food-grade enzymes in the market. Moreover, high-throughput purification and the identification analysis of these peptides are currently being studied to further understand the functionality and characterization of the bioactive peptides. This review mainly focuses on the novel bioactive peptides derived from fish protein hydrolysates from various fish wastes and byproducts. The hydrolysis conditions, source of hydrolysate, and amino acid sequence of these novel peptides are presented, along with their corresponding methods of analysis in purification and identification. The use of various enzymes yields novel peptides with potent bioactivities, such as antiproliferative, antimicrobial, antihypertensive, antiglycemic, antitumor, and antioxidative biological functions. The increasing interest in proteomics in marine and aquatic waste utilization continues due to these products' bioactivity and sustainability.

Keywords: sustainable development goals; food security; circular bioeconomy; fish waste; bioactive peptides; protein hydrolysate; protease; purification

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1. Introduction

Over the years, the steep rise in the world population has created immense pressure on the extraction of natural resources [1–5], including an overburden on the agricultural and food sector to feed people with stable nutritional diets [6–8]. Therefore, there is an urgent need to manage waste in a manner that solves the issues of food scarcity, food insecurity, and environmental pollution. The sustainable development goals of the United Nations propose to take stringent actions in the form of research and government policies to create a balance in these sectors. In the global trade setting, fish and marine products are considered highly perishable commodities and are commonly processed to maintain their nutritional components. In 2020, the global production of capture and aquaculture recorded that 157 million tons (89%) were utilized for human consumption, 20 million tons (16%) were

utilized for non-food uses, others were utilized as fish meal and fish silage (81%), and the remaining amount unaccounted for went to waste [9]. Due to the worldwide abundance of fish, it is considered one of the main protein sources for human consumption. A wide variety of products can be derived from fish, but most of the time, the fish byproducts are shown the least attention. Most fish products that are fresh and of optimum quality are high-priced commodities [10]. On the other hand, underutilized and abundant species are commonly subjected to traditional preservation techniques, which include salting, drying, and smoking, which lead to diversified product forms across continents and other parts of the world. These processing methods produce byproducts in high quantities, mainly composed of heads, fins, guts, and bones, and are often discarded as waste materials and thrown into landfills, oceans, and rivers [11].

The increasing demand for value-added products has led to a high production of fish waste that needs more attention to mitigate its negative effects on the environment [12,13]. The disposal of agro-fishery waste biomass accounts for 50% of the global aquaculture and processing industry production, and has been considered a global problem [14]. Significant actions are needed on the valorization of fish byproducts to improve the waste management and proper disposal of these fish scraps. The fish biomass including the skin, bones, head, and viscera are considered processing wastes produced by seafood processing activities which result in negative environmental and economic impacts [15]. However, fishery byproducts and discards are good sources of bioactive compounds and have a high potential for food applications and other industrial uses. At present, there is a growing interest in the utilization of fish wastes and discards that are generated from fishing activities and production [16], and most of them are considered not fit for human consumption.

Fish protein hydrolysates (FPH) are one of the high-quality products that can be derived from fish muscle protein. These are made from fish or fish byproducts by hydrolyzation, which yields a mixture of broken proteins and smaller compounds of peptides and amino acids [17]. The hydrolysis methods that are usually employed to extract fish proteins are chemical, microbial, and enzymatic processes. The chemical methods employ the use of alkalis and other solvents. However, in the recent past, deep eutectic solvents have also been recognized as "green" solvents for extracting novel proteins from fish discards such as skin, scales, bones, and viscera in an economical manner [18,19]. On the other hand, the enzymatic processes usually involve using pure commercial proteolytic enzymes. FPH is known to contain bioactive peptides and exhibit biological activities such as antioxidant and antimicrobial activities, angiotensin-I converting enzyme (ACE) inhibition, calcium-binding capacity, dipeptidyl peptidase (DPP)-IV inhibition, immunomodulation, and antiproliferative activity (Figure 1) [20]. Additionally, protein hydrolysates display functional characteristics, and each functional characteristic depends on the makeup, amino acid sequence, and size of the protein hydrolysate.

Efforts in the utilization and management of waste have been implemented worldwide, and the recovery of potent bioactive compounds is well studied. It can be noted from the literature that large amounts of these compounds from fish byproducts can be utilized and not be put to waste. The current review provides recent updates on the novel bioactive peptides derived from fish byproducts and discards using green solvents for industrial and nutraceutical purposes, which will allow a continuous supply of bioactive compounds and reduce environmental anthropogenic effects. In addition, various purification and identification techniques of novel bioactive peptides derived from fish byproducts are outlined and discussed. Additionally, this review paper will serve as baseline information for further research, specifically in agricultural and fishery waste valorization by utilizing low-prior biomass, using traditional and new emerging technologies.

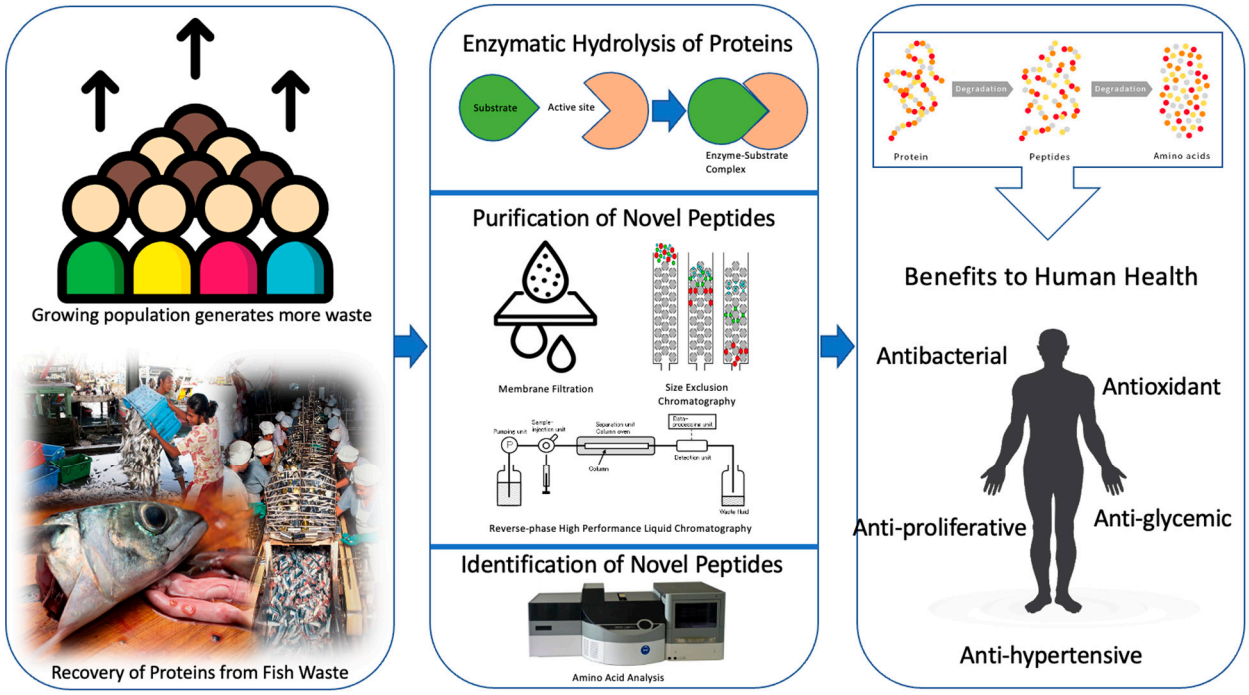

Figure 1. Production of novel bioactive peptides from fish protein hydrolysates with attractive health-promoting properties.

2. Status of Production Volume, Management, and Utilization of Fish Waste

The increasing population growth has been recorded for the past few decades and has been a major contributor to the utilization of non-renewable resources, resulting in many environmental issues [21–25]. Currently, effective and new technologies have been developed for sustainable approaches to address the increasing demand of the growing population worldwide [1,26,27]. In contrast, the increasing population increases plant and animal waste disposal [6,28]. The high biomass of waste being discarded, especially in water bodies, results in poor water quality and possibly contaminated fish resources. Awareness of the development of sustainable processes to address these problems is continuously growing, which has created interest in underutilized and often-neglected aquatic resources, including fish waste byproducts. It was estimated that more than 50% of the total fish catch is not consumed by humans [29], and is often produced as an animal feed, a good alternative ingredient used in various industrial and aquaculture products.

Most of the approaches for fish waste utilization include the recovery of protein and other essential biomolecules, and as aquaculture and agricultural fertilizers. A review on fish-waste-based fertilizers used in organic farming revealed that fish and fish waste are useful raw materials for developing good-quality solid and liquid fertilizers, and digestates [30]. On the other hand, fish waste is also utilized as an ingredient in food supplements, and recent updates on the sustainable recovery of omega-3 fatty acids in fish waste revealed several extraction methods. Specifically, fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been widely studied as potent ingredients in various food supplements with various health benefits [31]. Recently, fish waste has been widely studied for its protein recovery. The study by Araujo et al. [32] focused on the simultaneous production of fish hydrolysates, and utilized fish and fish byproducts as raw materials. The results revealed that the application of enzymes in the hydrolysis process resulted in the production of potent bioactive peptides and significantly reduced waste disposal by 79% in landfills; fish waste generation is reflected in Figure 2. In this context, studies on the utilization of fish byproducts and discards could have a significant contribution to the mitigation of environmental problems and future economic challenges.

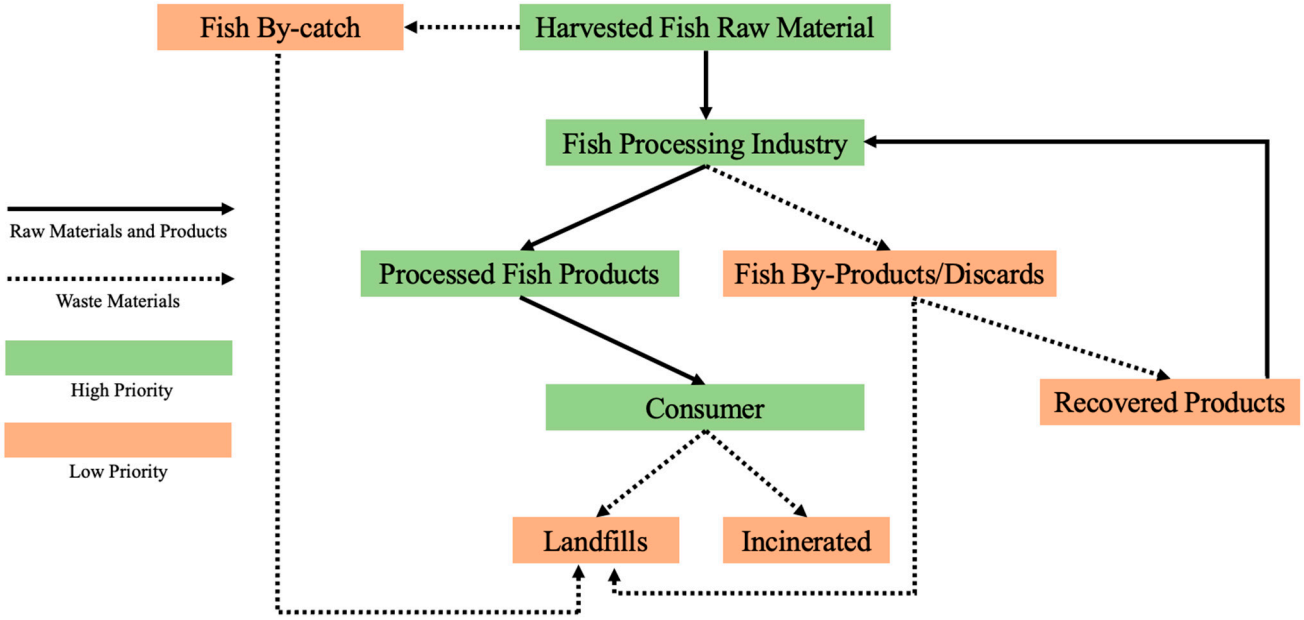

Figure 2. Schematic diagram of fish waste generation from fish harvesting and processing.

3. Application of Enzymatic Hydrolysis for Fish Protein Isolation

Large amounts of fish and fish byproducts are currently being discarded and utilized for low-quality and low-value products. Fish byproducts contain high-value bioactive molecules, found in the fish muscle. In addition, solid and liquid byproducts from fish are a good alternative source to utilize and recover potent bioactive molecules that may be present. One way to valorize this biomass is the use of enzyme technology, which is a widely practiced way to produce a broad spectrum of food ingredients with a wide range of applications.

The hydrolyzation of proteins from any source can be carried out via chemical (acid or alkali) treatment or the application of biochemical methods [15], and is achieved via proteolytic enzymes that are naturally occurring in fish muscle tissues. In addition, the treatment of commercial enzymes accelerates the process (enzymatic hydrolysis) and further breaks down larger-sized peptides into amino acids. Many studies in the literature on novel methods of fish protein hydrolysis have been published in scientific journals. A recent study was conducted by Esua et al. [33] on hybridizing plasma functionalized water and ultrasound pretreatment for enzymatic hydrolysis (HPUEH). The authors indicated that the optimized method accelerated the hydrolysis and revealed small-sized peptides with increased surface area and improved bioactivities. However, the use of these novel method needs further evaluation before they can be utilized for industrial-scale production. Another method developed by Tang et al. [34] utilized enzymatic hydrolysis by using commercial enzymes for fucoidan extraction from Gagome kelp and used β-glucosidase for the efficient degradation of cellulose, hindering the fucoidan release from the cell wall. However, both methods—as well as other novel methods available—have one major purpose: the extraction of potent bioactive compounds and further separation for improved functionality and bioactivities. In the hydrolysis of proteins, degradation or breakdown converts these molecules into smaller molecular structures, peptides, and amino acids, and smaller-molecular-mass peptides can be absorbed by the body of an organism faster than proteins [35].

Currently, many approaches to the enzymatic hydrolysis of proteins have been developed. Most methods follow the same principle with modifications [36]. Generally, the principle of hydrolysis involves the solubilization of a solid (fish) with water, mixed 1:1. This process needs further evaluation as to the ratio of water added to the solid, to ensure the suitable extent of protein breakdown and to avoid extra costs on drying unnecessary

water fractions from FPH in the drying process [15]. The extent of hydrolyzation is evaluated using the parameter named "degree of hydrolysis" (DH), which is commonly used and is an important factor related to the hydrolysis yield [37]. DH is measured and expressed as the ratio of broken peptide bonds to the total peptide bonds in the mixture per unit weight (Equation (1)): where DH is the degree of hydrolysis expressed in percent; *Pbr* is the number of broken peptide bonds; and *Ptot* is the total number of peptide bonds in the mixture.

$$\mathrm{DH} = \frac{Pbr}{Ptot} \times 100\% \quad (1)$$

The enzymatic hydrolysis of proteins has been extensively investigated in recent years, and products of this process have been utilized for industrial and nutraceutical purposes [7]. Marine products and byproducts, for example, have been studied and were found to have high contents of functional peptides and amino acids. In the process of enzymatic protein hydrolysis (EPH), endogenous or added enzymes can be used as catalysts to break down peptide bonds between amino acids [15]. The endogenous enzymes during EPH (also called autolysis) are naturally present in the digestive system of the fish, and it takes time to produce high amounts of cleaved peptides. According to Siddik et al. [38], the autolysis process is difficult to standardize and control since the production of enzymes from this source depends on factors such as age, season, species, diet, environment, etc.; traditionally, it is used as a method to produce fish sauce and silage. On the other hand, the use of commercial enzymes in EPH is known to have several benefits compared to autolysis or chemical hydrolysis and was found to have improved functionalities and bioactivities. Autolysis may result in the increased formation of undesirable metabolites, the formation of nitrogenous compounds, and the loss of freshness when there is poor handling and storage. While EPH using commercial proteases in the hydrolysis of food products was found to counteract the loss of functionality during the isolation of proteins [39], there is a need to carefully select the protease and control the hydrolysis time to maximize the potential of enzymatic hydrolysis to expand its range of potential food and nutraceutical applications [40]. Endogenous and exogenous enzymes in the fish processing industry are known to minimize and mitigate environmental pollution, and repeated efforts in the valorization of fish waste and discards have resulted in the production of various fish products with industrial applications [41].

3.1. Endogenous Enzymes

Enzymes that are naturally present or can be isolated from the digestive system of a fish are called endogenous enzymes, and they play a major role in digestion by breaking down carbohydrates [42]. After harvest, various biochemical changes occur in fish muscle, including the enzymatic hydrolysis of proteins. This breaks down muscle tissue and other connective tissue structures, resulting in decreased muscle integrity and changes in rheological properties [43]. In addition, autolytic processes in fish depend on the location of enzymes in the muscle, seasonal changes in enzyme concentration, and the synergistic effect of the enzymes in proteolysis [44]. Furthermore, endogenous proteases play a key role in the deterioration of most fin fishes [45–47]. Currently, studies are being developed to further assess the mechanism of action of these enzymes in various marine and aquatic fermented products [48].

A study conducted by Bu et al. [49] on the assessment of the endogenous proteases in fermented fish sauce revealed that four proteases, namely, serine protease inhibitor, trypsin inhibitor, aspartic protease inhibitor, cysteine protease inhibitor, and metalloprotease inhibitor, were present in the medium. The authors further explained a positive correlation of the endogenous protease activity on the antioxidant activities of fermented fish sauce. The fermentation of low-salt fish paste ka-pi-plaa, studied by Sripokar et al. [50], revealed high concentrations of endogenous proteases (mainly cathepsin-D) in the fermented product. The authors added that cathepsin-D is naturally occurring and a predominant proteinase participating in the autolysis process of beardless barbs. Fermented fish products are

commonly consumed as condiments and are also utilized as flavor enhancers that add nutrition to the final product [51]. Thus, the action of endogenous enzymes in proteolysis during fermentation results in the production of peptides and amino acids, which are somehow responsible for the development of taste and aroma unique to most fermented fish products.

3.2. Enzymes from Microorganisms

Exogenous enzymes are commonly utilized in the production of hydrolysates, silages, and other fish byproducts due to their capacity to increase the rate of production when added in appropriate levels [52]. Currently, endopeptidases from bacterial cultures are being produced and utilized as enzymes for starter cultures in fermentation, and in the production of fish byproducts, and these are known to release bioactive protein hydrolysates from fish [53]. Some known examples are commercially produced enzymes from *Bacillus licheniformis* and fungi *Aspergillus oryzae*, commonly known as Alcalase® 2.4 L and Flavourzyme®, respectively [54]. Additionally, enzymes such as Neutrase® (*Bacillus amyloliquefaciens*) and Protamex® (*Bacillus licheniformis* and *Bacillus amyloliquefaciens*) are also synthesized from bacterial cultures and are utilized in the industry. These enzymes have been studied for their mechanism of action in various food products, resulting in products with high yields and increased functionality. Shen et al. [55] investigated the properties of FPH from *Collichthys niveatus* hydrolyzed at different conditions using the abovementioned enzymes. Results from their study revealed that the Neutrase enzyme catalyzed the hydrolysis processes most effectively compared to other enzymes. Furthermore, the enzyme Neutrase revealed high contents of sweet and umami taste from amino acids. Novel bioactive peptides were also studied by Jemil et al. [56], derived from the enzymatic hydrolysate of Sardinelle muscle proteins by microbial proteases. In their study, *Bacillus subtilis* A26 proteases used as enzymes in the hydrolysis process revealed a total of 62 peptides and were found to exhibit antibacterial, antioxidant, and ACE-inhibitory activities. Some of the recent studies on novel bioactive peptides derived from the enzymatic hydrolysis of various FPHs from fish waste byproducts and discards are presented in Table 1.

Table 1. Fish byproducts utilized to produce novel peptides derived from enzymatically hydrolyzed fish proteins.

Byproducts	Species	Protease	Hydrolysis Conditions	Peptides/Amino Acid Fractions	References
Head	Squid (*Loligo formosana*)	Alcalase, Flavourzyme	3% E/S, 12.5 h, unadjusted pH	Arg-Glu-Gly-Tyr-Phe-Lys	[57]
Roe	Tuna (*Katsuwonus pelamis*)	Alcalase, trypsin	1% E/S, 4 h, 55 °C	Cys-Gly-Arg	[58]
Skin	Puffer fish (*Takifugu flavidus*)	Alcalase, neutral protease, pepsin	2000 U/g %E/S, 5 h, pH 8, pH 7, pH 2	Pro-Pro-Leu-Leu-Phe-Ala-Ala-Leu	[59]
Mixed waste	Round scad (*Decapterus maruadsi*)	Neutrase	0.3% E/S, 6 h, pH 7, 50 °C	KGFP, FPSV, FPFP, WPDGR	[60]
Cartilage	Siberian sturgeon (*Acipenser baerii*)	Alcalase	-	GPTGED, GEPGEQ, GPEGPAG, VPPQD, GLEDHA, GDRGAEG, PRGFRGPV, GEYGFE, GFIGFNG	[61]

Table 1. *Cont.*

Byproducts	Species	Protease	Hydrolysis Conditions	Peptides/Amino Acid Fractions	References
Fish milt	Skipjack tuna (*Katsuwonus pelamis*)	Alcalase	2% E/S, pH 9.5, 6 h, 55 °C	Tyr-Glu-Arg-Met, Tyr-Asp-Asp, Thr-Arg-Glu, Arg-Asp-tyr, Asp-Arg-Arg-Tyr-Gly, Ile-Cys-Tyr, Leu-Ser-Phe-Arg, Gly-Val-Arg-Phe	[62]
Mixed waste	Anchovies Family *Engraulidae*	Alkaline protease NS37071, neutral protease	pH 8, 55 °C, 5 h	Thr-Pro-Ser-Ala-Gly-Lys, Thr-Pro-Ser-Asn-Leu-Gly-Gly-Lys, Leu-Glu, Leu-Glu-Glu	[63]
Mixed waste	Deep-water Pink shrimps (*Parapenaeus longirostris*)	Savinase	40 U/mg E/S, pH 10, 55 °C, 3 h	SSSKAKKMP, HGEGGRSTHE, WLGHGGRPDHE, WRM-DIDGDIMISEQEAHQR	[64]
Scales	Grass carp (*Ctenopharyngodon idella*)	Alkaline protease BaApr1	1250 U/g E/S, 7 h, pH 9.5, 50 °C	Tyr-Val-Gln-Ala-Gly-Ala-Ala-Gly-Ala-Ala-Ala-His, Val-Lys-Leu-Tyr-Val-Leu-Leu-Val-Pro	[65]
Bones	Atlantic salmon	Trypsin	0.4% E/S, 40 °C, pH 8.0, 3 h	FCLYELAR	[66]
Viscera	Atlantic salmon (*Salmo salar*)	Pepsin	1% E/S, pH 2, 37 °C, 8 h	Thr-Pro-Glu-Val-His-Ile-Ala-Val-Aso-Lys-Phe	[67]
Skin	Nile tilapia (*Oreochromis niloticus*)	Properase E + multifect neutral	5% E/S, pH 8, 55 °C, 4.5 h	Glu-Gly-Leu, Tyr-Gly-Asp-Glu-Tyr	[68]

3.3. Bioactive Peptides' Production Using Animal-Based Enzymes

Bioactive peptides from fish protein hydrolysates can also be produced using enzymes derived from animals, such as pepsin [69]. These enzymes of animal origin are known to yield potent bioactive peptides derived from protein sources. There are wide applications of pepsin which have been applied in several fish hydrolysate production. A recent study conducted by Chel-Guerrero et al. [70], which focused on the antioxidant activities of Lionfish protein hydrolysates, revealed that using pepsin and pancreatin enzymes during hydrolysis resulted in large amounts of polypeptides with metal-chelating activities. Other bioactivities, such as the ACE-inhibitory activities of *Kawakawa* fish protein hydrolysates, were also investigated by Taheri and Bakhshizadeh [71], using the enzyme pepsin. The authors indicated that the recovered enzymes from the Skipjack tuna viscera produced good-quality hydrolysates with bioactivities. Moreover, Hassan et al. [72] studied the pepsin-derived visceral protein hydrolysate from *Pangasius* and showed prominent antioxidant activities compared to papain-derived hydrolysates. In another study conducted by Gao et al. [73], the peptide fractions derived from sturgeon fish muscles hydrolyzed by pepsin revealed the presence of anti-inflammatory peptides. It was further revealed that the peptides have anti-inflammatory effects against macrophages. It is interesting to note that the use of animal-derived enzymes to produce fish protein hydrolysates has high potential in industrial production, with high overall quality in the products.

4. Novel Peptides Derived from Enzymatic Hydrolysis of Fish Discards

Recent discoveries on novel peptides from fish and other aquatic products have greatly impacted the nutraceutical industry with varied applications. This made researchers more interested in studying the mechanisms of the peptides in vivo and in vitro. Recent studies have focused on the novel peptides from fish protein hydrolysates derived from fish byproducts and discards. The novel protein peptides from fish discards exhibit numerous bioactivities, including antioxidative, antimicrobial, antithrombotic, antigenotoxic, anti-obesity, anticarcinogenic, and antihypertensive activities, as well as other biological functions such as mineral binding and immunomodulatory activities, which are quite beneficial to human health [74,75].

4.1. Antiproliferative Peptides

Bioactive peptides with antiproliferative activities are considered novel prospects utilized to develop cancer drugs, considering the minimized side effects and cost. Antiproliferative peptides inhibit the growth of cancer cells in various ways, including disrupting the cytoplasmic membrane through micellization, inducing apoptosis, and interacting with the gangliosides on the cell surface. Shaik and Sarbon [74] discussed the antiproliferative peptides derived from fish protein hydrolysates and their development strategies. The authors indicated different approaches to isolating and purifying these enzymes and new approaches to the characterization of the peptides. However, most of the analyses were carried out in vitro and need confirmatory results through in vivo analysis.

Bioactive peptides, especially those from fish hydrolysates, have the capacity to lessen oxidative stress due to reduced reactive oxygen species (ROS), which in turn inhibits genetic alterations such as mutation and chromosomal aberrations, which are typically important in carcinogenesis. A study conducted on the antiproliferative activity of protein hydrolysates from fish byproducts tested human colon and breast cancer cells [76]. It was revealed that FPH from the skin, bones, head, and viscera from different species significantly inhibited the growth of cancer cells. Furthermore, the authors suggested that the isolation of the responsible peptides for growth inhibition should be carried out and integrated into food supplements. Hamzeh et al. [77] studied the antiproliferative and antioxidative activities of cuttlefish (*Sepia pharaonic*) protein hydrolysates, and it was revealed that the FPH inhibited the growth of the tested cancer cells (MDA-231 and T47D), with growth inhibition of 78.2 and 66.2%. The work of Yu et al. [78] focused on *Cyclina sinensis* protein hydrolysates (CSP) for the production of a novel peptide with antiproliferative pentapeptides that can induce the apoptosis of prostate cancer cells. The authors further stated that the developed hydrolysates from *C. sinensis* significantly inhibited the growth of DU-145 cells. Furthermore, the peptides from CSP may represent a therapeutic and nutraceutical agent for treating prostate cancer patients. These novel discoveries in finding the solutions to curing various cancer diseases are very interesting, and derived peptides have a high potential to be utilized in these treatments. In this regard, it can be noted that there is evident proof that aquatic and marine resources are good natural sources for medicinal purposes.

4.2. Antimicrobial Peptides

Most bioactive peptides derived from fish protein hydrolysates have been shown to have a broad range of deteriorative actions against diverse microbes, including bacteria, fungi, viruses, and protozoa [79,80]. They possess a wide range of antifungal and antimicrobial properties. Various peptides from fish with antimicrobial activities have been studied widely in the past decades for their good usability in the nutraceutical industry. The isolation of these novel peptides was also studied, as in the work of Park et al. [81], who identified novel bioactive peptides that were obtained from mudfish (*Misgurnus anguillicaudatus*), which they named *misgurin*. The peptide was found to cause damage to the cell membrane in various microorganisms and has high potency. Additionally, Tang et al. [82] studied anchovy hydrolysates and identified a novel peptide with a membrane-disruptive property. Furthermore, the previous authors indicated that peptide Pep39 disrupted the

E. coli membrane and suggested that the mechanism of Pep39 includes cytoplasmic membrane damage. Zhang et al. [83] characterized a novel peptide with antibacterial properties, which was isolated from hemoglobin alpha in the liver of Japanese eels. The authors discussed that peptides with concentrations of 11 μM exhibited stronger activity against the pathogenic bacterium *Edwardsiella tarda*. Contrary to this, Seo et al. [84] studied the skin of yellowfin tuna (*Thunnus albacares*) and was able to characterize a novel peptide with antimicrobial properties. The authors indicated that the peptide has an amino acid sequence of YFGAP, and showed its potent activities in inhibiting the growth of Gram-positive bacteria, such as *B. subtilis*, *M. luteus*, and *S. aureus*. These novel antibacterial peptides can be found in various hydrolysates derived from marine or aquatic resources, and they have great potential in nutraceutical applications.

4.3. Antioxidative Peptides

One of the crucial roles of bioactive peptides is their antioxidant activity, and this can be attributed to specific biological functions such as scavenging free radicals, the inhibition of lipid peroxidation, and metal ion chelation [85]. Novel bioactive peptides from fish hydrolysates obtained from fish byproducts have been widely studied, as in the work of Najafian et al. [86], in which three novel peptides were isolated from patin (*Pangasius sutchi*) myofibrillar protein hydrolysates. After purification and testing for their antioxidative properties, the peptides exhibited the highest antioxidant activity. In terms of muscle protein hydrolysates, Bashir et al. [87] identified novel antioxidant peptides from mackerel (*Scomber japonicus*) muscle protein hydrolysates. Furthermore, the authors characterized the peptide ALSTWTLQLGSTSFSASPM as having the highest DPPH scavenging activity, and the LGTLLFIAIPI peptide to have the highest SOD-like activity.

Zhang et al. [68] investigated the novel antioxidant peptides of gelatin skin hydrolysates from tilapia (*Oreochromis niloticus*). The authors showed that the amino acid sequences of peptides Glu-Gly-Leu and Tyr-Gly-Asp-Glu-Tyr were found to have high hydroxyl radical scavenging activities and suggested that using properase E enzyme could yield these antioxidant peptides. Alternatively, Saidi et al. [88] investigated the valorization of tuna processing waste biomass and observed that the four novel antioxidant peptides, identified as Tyr-Glu-Asn-Gly-Gly, Glu-Gly-Tyr-Pro-Trp-Asn, Tyr-Ile-Val-Tyr-Pro-Gly, and Trp-Gly-Asp-Ala-Gly-Gly-tyr-Tyr, exhibited good scavenging activity against hydroxyl radicals.

In a research study, a novel heptapeptide from mackerel byproduct hydrolysates was identified by Kim et al. [89], who characterized the heptapeptide TCGGQGR with high antioxidant activities and potential functional fertilizer properties. It is interesting to note that fish byproducts obtained from the fish processing industry have high potential as major sources of peptides with antioxidant properties, and the valorization of these discards is a necessary step to reduce the waste in this industry.

4.4. ACE-Inhibitory Peptides

A well-known mechanism of antihypertensive peptides is the angiotensin-I-converting enzyme (ACE) inhibition, and these compounds are derived from food-related proteins which have been studied for their potential in the management of hypertension [90]. Novel ACE-I peptides from marine and aquatic byproducts are also being studied. Krichen et al. [64] identified a novel ACE-inhibitory peptide from shrimp waste hydrolysates hydrolyzed by Savinase, revealing that the peptides exhibited high antihypertensive activities with high affinity towards ACE. Aissaoui et al. [91] studied protein hydrolysates from red scorpionfish (*Scorpeana notata*) byproducts and identified novel peptides, namely, Gln-Gln-Pro-His-Ser-Arg-Ser-Lys-Gly-Phe-Pro-Gly-Pro, Gly-Gln-Lys-Ser-Val-Pro-Glu-Val-Arg, and Val-Glu-Gly-Lys-Ser-Pro-Asn-Val. Additionally, the authors stated that the abovementioned peptides showed high ACE-I-converting-enzyme-inhibitory activity. Similarly, in another study, two novel peptides were identified from the muscle protein hydrolysates of red scorpionfish, with amino acid sequences of Leu-Val-Thr-Gly-Asp-Asp-Lys-Thr-Asn-Leu-

Lys and Asp-Thr-Gly-Ser-Asp-Lys-Lys-Gln-Leu; they are mainly composed of hydrophilic amino acids and show good antioxidant and ACE-I activities [92].

Chen et al. [93] purified and characterized a novel ACE-inhibitory peptide derived from grass carp protein hydrolysates hydrolyzed by alcalase. The authors indicated that the identified Val-Ala-Pro (VAP) is the first reported food-derived tripeptide and was observed to have excellent ACE-I activity and unique biochemical properties. For the in vivo analysis of the mechanisms of these antihypertensive peptides, Lee et al. [94] investigated the antihypertensive effects of tuna frame protein hydrolysates in spontaneously hypertensive rats and observed that the isolated peptide with the amino acid sequence Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro was a noncompetitive inhibitor against ACE. The authors further presented that oral administration of the said peptide in spontaneously hypertensive rates significantly decreases systolic blood pressure. Zheng et al. [95] recently identified novel ACE-I peptides from muscle hydrolysates of skipjack tuna, where alcalase was used in hydrolysis to obtain Ser-Pro and Val-Asp-Arg-Tyr-Phe peptides with high ACE-I activities. Based on the novel discoveries of antihypertensive peptides, it can be noted that byproducts and discards from aquatic and other marine species are good sources of ACE-I peptides and could be relevant to the nutraceutical industry, and for other industrial purposes as an anti-hypertensive component in functional foods.

4.5. Dipeptylpeptidase-IV (DPP-IV)-Inhibitory Peptides

DPP-IV inhibitors such as peptides play a key role in glycemic regulation (Nongonierma and Fitzgerald 2017), and this biological function of peptides is considered important in the latest developments in food-protein-derived peptides. There are few intervention studies of the novel food-protein-derived DPP-IV-inhibitory peptides, such as the work of Jin et al. [96], which identified novel DPP-IV-inhibitory peptides from Atlantic salmon skin collagen hydrolysates hydrolyzed by trypsin; they observed that the novel peptides inhibited the DPP-IV enzyme activity. The authors further identified the peptide LDKVFR to have the highest DPP-IV-inhibitory activity, and it was observed to bind to DPP-IV through hydrogen bonds and hydrophobic interactions. Likewise, Kula et al. [97] investigated the myofibrillar proteins of *Trachinus draco* (greater weever) and identified novel multifunctional peptides with high ACE-I- and DPP-IV-inhibitory, antioxidant, and metal-chelating activities. The authors further indicated that two peptides have multifunctional properties, and after de novo sequencing methods, it was observed that peptide Phe-Pro-Gly-Asp-His-Asp-Arg exhibited DPP-IV-inhibiting, metal-chelating, and antioxidant activities. To date, there are few studies on the specific role of food-protein-derived DPP-IV-inhibitory peptides specifically derived from fish byproducts and wastes, and the key roles of DPP-IV-inhibitory peptides in the regulation of glycemia in humans should be further explored.

5. Characterization of Novel Bioactive Peptides

The purification and characterization of peptides involve various methods that need accuracy in data analysis, since the composition of peptides has 3–20 complex amino acid residues, and their composition and sequences are the basis of their bioactivities [98]. The importance of the identification of these bioactive peptides provide researchers with an idea of the structural properties of peptides released by enzyme hydrolysis. The advanced and recent technologies developed in the purification and identification of novel bioactive peptides from fish wastes and discards are being increasingly used (Figure 3).

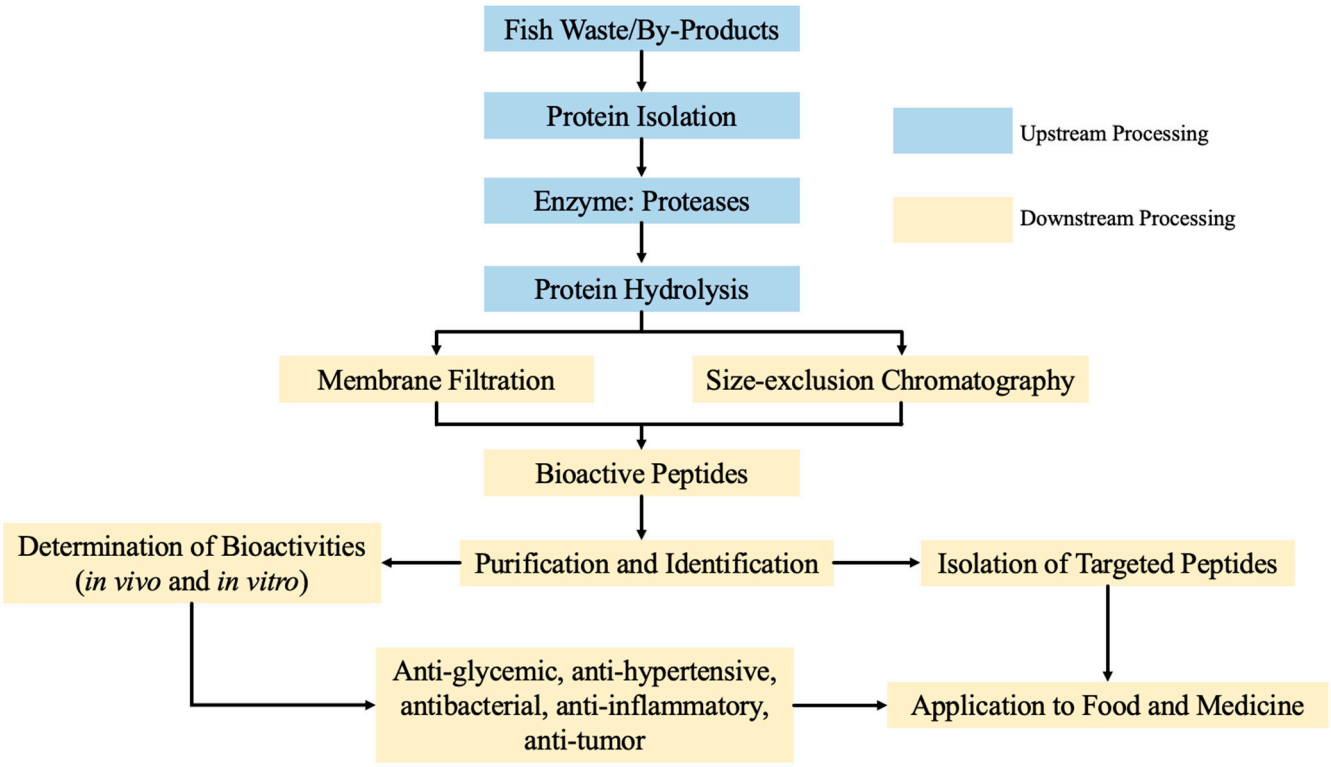

Figure 3. Schematic diagram of isolation and purification of peptides from fish waste and byproducts.

5.1. Purification and Identification of Peptides Using One-Dimensional Separation Systems

Nowadays, various techniques are being used to purify and identify bioactive peptides from marine and aquatic byproducts. High-throughput equipment with high accuracy, selectivity, and detection are used to study bioactive molecules such as fatty acids, enzymes, peptides, and amino acids. The recent developments in analytical techniques applied to the purification and identification of peptides and amino acids derived from marine and aquatic byproducts and discards are explained.

5.1.1. Membrane Fractionation (Ultrafiltration and Nanofiltration)

The process of protein ultrafiltration (UF) involves using a pressure-driven membrane to concentrate or purify proteins in aqueous solutions [99]. The typical pore sizes of UF membranes are in the range of 10–500 Å, and the rate of filtration of components depends on their response to the given pressure driving force. In separating peptides from non-hydrolyzed proteins, the UF method can be considered, and UF membranes such as molecular weight cut-off (MWCO) with a size of 20 kDa have been studied to retain proteins effectively [100]. Chabeaud et al. [101] compared various UF membranes for fractionating a fish protein hydrolysate, and the authors suggested that using UF membranes can improve the bioactivity of the tested peptides with sizes smaller than 7 kDa by fractionating some specific-molecular-weight peptide classifications. Recently, modifications have been made in the use of UF membranes, such as in the work of Roslan et al. [102], who used a multilayer UF membrane to fractionate tilapia byproduct protein hydrolysates and observed that peptide selectivity could be improved using 5/5 multilayer membranes. Likewise, Pezeshk et al. [103] investigated the fractionation of protein hydrolysates from fish waste by using UF and were able to separate peptides into four fractions (<3, 3–10, 10–30, and >30 kDa), which showed good antimicrobial and antioxidant properties.

The use of nanofiltration (NF) has been widely utilized in the field of biological research, with a focus on the combination of nanoparticles and biological compounds such as proteins to form a more functional and hybrid system [104]. Some researchers emphasized that the challenge for fractionation and purification is separating these hybrid

materials from unbound free biomolecules. Some work has been published on combining UF and NF, especially in the field of biochemistry. Fish fillet hydrolysates were fractionated by Vandanjon et al. [105] using UF coupled with NF, showing that fractions with a size of 3000 Da had the highest yield. Furthermore, the authors suggested that the refinement of methods should be conducted via purification in diafiltration mode to achieve the most active fractions. Meanwhile, Picot et al. [106] investigated the effect of UF and NF on fish protein hydrolysates produced from the processing industry and observed that the successive fractionation of peptides on UF and NF membranes was able to select concentrations of selected sizes of peptides. It is interesting to note that the combination of multiple membranes to classify peptides according to size could significantly improve the selectivity of the filtration system used and would yield high-quality peptides with improved bioactivities.

5.1.2. Gel Filtration (Size-Exclusion Chromatography)

Gel filtration is a widely used technique that determines the size of proteins, and it is considered an effective method that allows the separation of proteins and other biological molecules with a high yield [74,107]. This separation technique is widely utilized in studying fish proteins, such as in the recent work of Hu et al. [108], in which the authors purified and identified a novel ACE-I peptide from *Lepidotrigla microtera*, using gel filtration chromatography (Sephadex G-15 column) as a separation technique, and five peptide fractions with potent activities were separated. On the other hand, the authors Pan et al. [109] also utilized gel filtration chromatography (Sephadex G-15 column) to purify a novel ACE-I peptide from *Enteromorpha clathrata* or Ulva seaweed protein hydrolysates. Furthermore, the authors identified the peptide as Pro-Ala-Phe-Gly and indicated that the novel peptide was non-competitive in its inhibitory kinetic mechanism. Wang et al. [110] purified five novel peptides from spotless smooth-hound (*Mustelus griseus*) muscle, using gel filtration as one of the consecutive chromatographic methods employed for the ethanol-soluble proteins with potent antioxidant activities. Besides utilizing the separation method in protein fractionation, the work of Maalej et al. [111] utilized gel filtration (Sephadex G-100) as a consequent method for the purification of digestive enzyme alpha-amylase from blue crab (*Portunus segnis*) viscera hydrolysates. It is important to note that peptides and enzymes from marine and aquatic resources could be purified using separation techniques such as gel filtration, and this is a necessary additional method prior to peptide characterization and identification. The aid of this method could provide purified biological peptides without compromising their functionality.

5.1.3. Reverse-Phase High-Performance Liquid Chromatography

The commonly used separation technique that is versatile and usually employed in chemical analysis is called high-performance liquid chromatography (HPLC), and the reverse-phase HPLC is a more favorable separation technique due to its simplicity, versatility, and wide scope, since it can handle diverse compounds in terms of polarity and molecular mass [112]. Studies on the improvement in this separation technique concern the optimization of amino acid resolution in RP-HPLC chromatograms. The RP-HPLC separation technique is usually employed to purify potent peptide fractions from fish hydrolysates. The study by Robert et al. [113] characterized peptide fractions from tilapia (*Oreochromis niloticus*) byproduct hydrolysates and utilized RP-HPLC to purify the peptides with antimicrobial activities from tilapia hydrolysates. Girgih et al. [114] isolated peptide fractions from salmon (*Salmo salar*) hydrolysates using RP-HPLC and observed that the isolated peptide fractions differ in hydrophobicity, yet the fractionation that was employed improved the free radical scavenging properties of the salmon peptides. Additionally, the work by Lan et al. [115] characterized peptides with ACE-I properties and utilized a rapid separation technique by applying a magnetic field, and they further identified the peptides using RP-HPLC. The authors further stated that the affinity interaction used in the initial purification of the peptides, followed by RP-HPLC, resulted in an effective purification

of ACE-I peptides from food sources. Based on these studies, it can be noted that the use of RP-HPLC as a one-dimensional separation and purification method, or combined with other methods, could be used to purify other bioactive peptides from fish hydrolysates and other sources.

5.2. Amino Acid Analysis

Amino acid analysis is widely used to determine the amino acid content of amino acids, peptides, and protein-containing compounds (Table 2) [116]. Amino acid analysis is usually accomplished by cation exchange, RP-HPLC to fluorescence, or the absorbance detection of pre-column or post-column derivatized amino acids [117]. Amino acids in food products can be in free form or in bound form, such as building blocks of proteins, and identification techniques for amino acids include HPLC and GC-MS and have been used in tandem with capillary electrophoresis MS and Ultra HPLC-MS coupled with detectors [118]. Additionally, Otter [118] recommended this series of methods for amino acid analysis in food: (1) protein hydrolysis, (2) chromatographic separation, (3) detection and quantification. Roslan et al. [119] characterized fish protein hydrolysates from tilapia (*Oreochromis niloticus*) and utilized a Waters Pico Tag Amino Acid Analyzer System, in which the samples were hydrolyzed and derivatized prior to analysis. With this, the suggested amino acid analysis could be employed in peptides derived from hydrolyzed fish byproducts to further determine the amino acid sequence to better understand its functionality.

Table 2. Analytical methods used on purification and identification of bioactive compounds derived from marine and aquatic byproduct and discard hydrolysates.

Hydrolysate Source	Sequence of Purification and Identification Methods	Bioactive Compound	References
Tuna skin (*Katsuwonus pelamis*)	UF–GF–RP-HPLC–AA sequence analysis–MW analysis	Collagen, antioxidative peptides	[120]
Oyster meat (*Crassostrea hongkongensis*)	UF–GF–RP-HPLC–LC/MS/MS	Antioxidative peptides	[121]
Lizard fish muscle	MA-IML separation, RP-HPLC–AA sequence analysis–MD	ACE-I peptide (VYP)	[112]
Tuna muscle (*Thunnus albacares*)	UF–GF–HPLC-MS/MS–MD	Antioxidative peptides (ACGSDGK)	[122]
Ribbonfish	UF–NF–RP-HPLC–AA sequence analysis–LC-MS/MS	ACE-I peptides	[123]
Shortfin scad (*Decapterus macrosoma*)	UF–GF–RP-HPLC–MD	ACE-I peptides	[124]

6. Outlook and Future Perspectives

Waste management is a continuous effort by every country around the world. The practice of recycling, reusing, and treating these wastes in the form of liquids and solids is quickly gaining pace because of the continued development of government policies. Countries have been supporting studies on the valorization of waste biomass. However, on the industrial level, regarding the treatment of wastes, there is average to minimum attention paid to the recovery of potent compounds and other materials, and there is high cost of the treatment process, which cannot be overlooked. The treatment of fish wastes generated by the processing industry is expected to promote a higher economy, as well as a sustainable and safe environment as part of the circular economy strategy. Government policies and implementing bodies should implement research-based approaches for the valorization of liquid and solid waste generated by the fish processing industry. Governments should provide a holistic framework to encourage and promote the processing industries and small-scale processors for fish waste treatment and value addition through chemical and mechanical methods by converting these wastes and byproducts into valuable products, for example, through the development of hydrolysates with wide applications. Moreover,

researchers should be encouraged to develop new and innovative approaches to fish waste management by optimizing the utilization of these wastes to produce new, potent products in a cost-effective and eco-friendly manner. Through this, consumer acceptance will be influenced while upholding the fish processing industry in the economy.

7. Conclusions

The current review provides an insight into the novel bioactive peptides that could be generated from fish protein hydrolysates from fish wastes hydrolyzed by commercial enzymes, which present these novel peptides as possible ingredients for food supplements. The valorization of fish waste has the potential to provide a good source of potent bioactive compounds that may help in lowering environmental pollution as well. Developing cost-effective and eco-friendly methods for the production, purification, and identification of these bioactive peptides needs more attention since these various analytical methods require high cost and are time consuming. An effective method for the valorization of waste includes the pretreatment of waste to reduce contamination prior to processing, and the use of proteases as catalysts for the hydrolysis of proteins with a higher yield of potent peptides. Furthermore, investigation of the mechanism of action of these bioactive peptides requires in vivo studies and thorough investigation to prove their bioactivity. There are still different forms of waste from different marine and aquatic sources that need investigation due to the vast number of resources that are being produced and often accumulated as wastes due to their abundance and low demand.

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Abbreviations

AA	Amino Acid
GF	Gel Filtration
LC	Liquid Chromatography
MS	Mass Spectrometry
MA-IML	Metal Affinity Immobilized Magnetic Liposome
MD	Molecular Docking
RP-HPLC	Reverse-Phase High-Performance Liquid Chromatography
SSF	Solid-State Fermentation

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