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Biological activities of peptides obtained by pepsin hydrolysis of fishery products

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Abstract

The fishing industry generates tons of waste of great intrinsic value due to its high content of biomolecules such as proteins. The processing of proteins can result in products with high nutritional, pharmacological, and technological interest due to the peptides that can be derived from them. This review work compiles the investigations that have performed on the production of peptides from proteins of fish origin using pepsin as catalyst from the corresponding hydrolytic reaction, with special emphasis on the description of each of the reported biological properties, as well as on some uses that have been explored for these peptides. This work may be useful to promote new research involving the use of pepsin in the production of bioactive peptides from fishery products, as well as for the development of mechanisms that allow their use in different industrial processes.

Keywords: proteolysis, biomass recovery, bioactive peptides, fish proteins

Introduction

Fisheries production exceeded 170 million tons worldwide during 2018, which represented a significant increase in their economic activity [1]. The growth of this industry is mainly due to an increase in per capita consumption of fish in developed countries [2]. This phenomenon in turn, has led to an increase in the volume of by-products, which represent approximately 50% of the complete fish, and that are generally discarded as waste (causing a negative impact on the environment) or used to obtain products with low added value [3,4] such as fish meal and fish oil [5]. However, this situation can be reduced through the implementation of biotechnological processes that allow the use of these residues to obtain biomolecules of great commercial interest, such as enzymes, polyunsaturated fatty acids, essential amino acids or peptides with biological activities [6,7].

The high content (10-25%) [8] of proteins in fish muscles and by-products (heads, viscera, cuttings, roe, frames, clippings, skins and spines) (8-35%) [9] together with their high diversity (due to the presence of different groups of proteins such as myofibrillar, stromal and sarcoplasmic [10–13]), gives fish by-products a great potential for obtaining bioactive peptides, which have aroused special interest in the food and pharmaceutical industry, due to their important and diverse biological properties [14]. As it can be seen, this activity allows adding value to raw materials with high protein content and low commercial value [9].

The process to produce peptides with bioactive properties from fish and by-products consists of different stages (Fig. 1). First, a pretreatment of the raw material is carried out, to form a homogeneous mixture (water-ground fish parts) with a low fat content [15]. The second stage of the process is known as hydrolysis, which can be carried out chemically (in

52 acidic or alkaline media) or using proteases of animal, vegetable or microorganism origin
53 [16]. Chemical methods are not generally recommended because they damage the quality of
54 the final product [8] and are not environmentally friendly [17]. The third step is the
55 fractioning and purification of the peptides, which is carried out because most scientific
56 reports indicate a greater biological activity of smaller peptides with variable sizes between
57 3 and 20 amino acids [18–20], although most sequences are between 2 and 4 amino acids
58 long [16]. This stage is usually carried out using different techniques such as ultrafiltration
59 membranes [21], electrodialysis [22], electrodialysis with ultrafiltration membrane [23,24]
60 or different chromatographic techniques, among which size exclusion, ion exchange and high
61 resolution liquid stand out [25]. Since the biological properties of the peptides are expressed
62 by the peptide when it is incorporated to the protein [26], the analysis of the biological
63 properties is carried out in several moments of the process. An initial analysis is performed
64 after the hydrolysis process and an additional one is done for each purification step product.

65 **Fig. 1. Should be here**

66 Bioactive peptides have the ability to manage, prevent or treat certain diseases such
67 as diabetes [27], cancer [28] or cardiovascular disease [29], and also play an important role
68 in the development of functional foods [30,31], due to their biological properties such as
69 antioxidant, immunoregulatory, antimicrobial, anti-inflammatory, anti-allergenic activities,
70 among others [32]. The bioactivity of peptides depend on their chemical structure, size and
71 amino acid composition [33], that are defined by the protein from which they were cleaved
72 and the method used for protein hydrolysis [34]. Particularly, when enzymatic hydrolysis is
73 used for the release of peptides, the enzyme used during the process plays a fundamental role
74 in the quantity and properties of the peptides released [35], since depending on the enzyme,

the protein will be cut at different bonds [14], that is to say that the selectivity and specificity that each enzyme has towards certain bonds, will propitiate that different peptide fractions are obtained from the same protein when using different enzymes. This diversity of peptides that can be obtained using different protein sources and employing different enzymes has led to the development of several investigations for the recovery of biological peptides using different proteolytic enzymes among which are Alcalase [36], papain [37], ficin [38], bromelain [39] or pepsin [40].

Pepsin features

Pepsin (BRENDA:EC3.4.23.1) is one of the three main proteolytic enzymes in the digestive system, and is found in gastric juices at a concentration of 400 mg/L. Pepsin (human and porcine) is produced in the mucosal lining of the stomach in an inactive form (pepsinogen) and is converted to its active form (pepsin) through proteolytic degradation [41]. Pepsinogen is a molecule that has two lobes (N and C terminal), that are stable at alkaline pH; however, when the pH decreases below 5, the N-terminal lobule is removed, releasing active pepsin with a molecular weight between 34 and 37 kDa [42,43]. It has been reported that the behavior of this enzyme in acidic media is favored by the existence of a phosphoryl group covalently attached to Ser68. This causes porcine pepsin to have a net negative charge even at acid pH values [44], explaining why this enzyme exhibits high efficiency in acidic environments (pH 1-3). The pepsin specificity is mainly influenced by the amino acid residues at position P1 and P1′ with a preferential cleavage in hydrophobic residues, such as phenylalanine, tryptophan or tyrosine [45–47].

Pepsin, mainly of porcine, bovine and microbial origin, is one of the most important industrial enzymes, accounting for approximately 60% of commercially marketed enzymes

[48]. This enzyme has application in multiple industries such as leather tanning, detergent, agrochemical, food and pharmaceutical industry [43]. In addition, pepsin is widely used in the production of peptides showing high antioxidant [49], antihypertensive [50], antimicrobial [51] and many others biological activities and functionally properties [52–57]. In this regard, it is particularly important to mention that a treatment with pepsin has been observed to reduce the allergenicity of certain proteins, and this is closely related to one of the functions indicated for this enzyme in the human digestive system [58,59], since this enzyme has an important role in reducing the risk of allergenic peptides reaching the intestinal lumen [60]. In this way, pepsin can be used in the production of hydrolysates or peptides, which in addition to having biological activities, have reduced allergenicity [61]. Furthermore, considering that this enzyme has an affinity to cleave the bonds involving hydrophobic amino acids [62] the released peptides will have hydrophobic amino acids in their structure (Leucine, isoleucine or Valine). These peptides have better antioxidant activity [63] and ACE-inhibitory activity [64] than other peptides. This makes pepsin seem like a protease of great interest for obtaining peptides with strong biological activities.

Thus, the objective of this review is to compile the literature indexed in Scopus, about obtaining peptides with bioactive properties from protein of fish origin, using pepsin as a hydrolytic catalyst.

Bioactive properties of peptides

Antioxidant capacity

The excess of free radicals is a problem of great consideration both in the medical and food industries, since these compounds affect the quality of food, shortening its useful life and promoting oxidative stress that can lead to the appearance of different chronic diseases

[65]. To counteract the negative effects of free radicals, the food industry has proposed the use of synthetic antioxidants such as t-butylhydroquinone, propyl gallate and butylhydroxyanisole, which negatively affect human health when consumed in high doses [14,66]. On the other hand, food-derived antioxidant peptides have no adverse effects compared to chemical synthesized antioxidant compounds[67,68]. Peptides with antioxidant activity are capable of reducing oxidative stress and lipid oxidation caused by free radicals [67]. For this reason within its potential applications are the development of health-promoting foods and the maintenance of the quality and safety of food products [69].

In general, it is known that the main mechanisms by which antioxidant peptides inhibit oxidation are through scavenging free radicals, inactivation of reactive oxygen species, transition metals, chelation of pro-oxidative compounds or reduction of hydroperoxides [70]. However, the antioxidant capacity of peptides is not yet known exactly, because this biological property is generally measured using *in vitro* methods (designed for the analysis of samples of plant origin) which do not allow accurate results to be obtained with respect to *in vivo* activity [71,72], which generates a halo of greater uncertainty about the true antioxidant capacity of the peptides. Due to the above, most of the research carried out in obtaining and characterizing bioactive peptides tries to include at least two techniques for the evaluation of antioxidant activity. Among the most used methods are Total Radical Trap Antioxidant Parameter (TRAP), Oxygen Radical Absorbance Capacity (ORAC), and Carotene Bleaching Assay [73], 2,20 -Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method, Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, hydroxyl radical (OH) scavenging activity, superoxide anion (O_2) radical scavenging activity and superoxide dismutase (SOD) [74,75] The above methods are based on the antioxidants

144 reaction with free radicals by hydrogen atom transfer (HAT) or single electron transfer
145 mechanism (SET); or the combination of both HAT and SET mechanisms [69]. For example,
146 the FRAP method is based on the reduction of ferric- tripyridyltriazine $[FeIII(TPTZ)]^{3+}$
147 (light-yellow color) to ferrous-tripyridyltriazine $[FeII(TPTZ)]^{2+}$ (intense blue color) under
148 acidic conditions (pH 3.6) [72] (Equation 1).

149 Equation 1: $[Fe^{III}(TPTZ)]^{3+} + ArOH \rightarrow [Fe^{II}(TPTZ)]^{2+} + ArO^{\bullet} + H^{+}$

150 The DPPH method evaluates the antioxidant capacity of biomolecules against the
151 $DPPH^{\bullet}$ radical. This method has the advantage of identifying antioxidant species that use
152 both HAT (equation 2) and SET (equation 3) as an antioxidant mechanism [76]

153 Equation 2: $DPPH^{\bullet} + ArOH \rightarrow DPPH - H + ArO^{\bullet}$

154 Equation 3: $DPPH^{\bullet} + ArOH \rightarrow DPPH - H + [ArO]^{\bullet+}$

155 In the ABTS method, the ABTS•+ radical is reacted with ammonium or potassium
156 persulfate for 16 hours before adding the antioxidant agents, which stabilize ABTS•+ by
157 donating an electron (equation 4) [77].

158 Equation 4. $((NH_4)_2)S_3O_3 + ABTS \rightarrow ABTS^{\bullet+}ArOH \rightarrow ABTS + ArO^{\bullet}$

159 The ORAC method is based on the loss of fluorescein from the target molecules when
160 they are attacked by peroxyl radicals, a loss that is delayed by the presence of antioxidants in
161 the medium, which quench the peroxyl radicals by transferring hydrogen atoms (equation 5)
162 or by the sum of radicals (equation 6) [77].

163 Equation 5. $ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$

Equation 6. $ROO^{\bullet} + A^{\bullet} \rightarrow ROO - A$

As it will be seen in the studies presented below, it is common to use two or more of the methods described, in order to characterize the antioxidant activity of the peptides obtained.

The work carried out by Chalamaiah et al [78], showed how, hydrolysates could be obtained from eggs of *Labeo rohita* roe using pepsin as a proteolytic enzyme with capacity values for scavenging $ABTS^{\bullet}$ and $DPPH^{\bullet}$ radicals by ≥ 80%, as well as an ability to reduce more than 55% the Fe^{3+} ion presence [78]. In another work, six different enzymes (Alcalase, Flavourzyme, Neutrase, pepsin, Protamex and trypsin) were used to hydrolyze salmon dorsal fins, producing a hydrolysate with great activity to stabilize the $DPPH^{\bullet}$ radical [79]. Pepsin hydrolysis resulted in a hydrolysate with the highest antioxidant activity, from which a peptide (Phe-Leu-Asn-Glu-Phe-Leu-His-Val) responsible for a large percentage of the antioxidant activity of the entire product was purified and sequenced [79]. Pepsin also proved to be better than papain for producing hydrolysates from *Rastrelliger kanagurta* (Indian mackerel) backbone with antioxidant activity, property evaluated in terms of ability to scavenge $DDPH^{\bullet}$ (46%) and superoxide (58.5%) radicals, ability to reduce Fe^{3+} and inhibit lipid oxidation [5].

Fish by-catch can be used in the production of hydrolysates with antioxidant properties, as it was done with the muscle of *Decapterus maruadsi* using pepsin as a hydrolyzing agent. The obtained hydrolysate presented activity to quench the $DPPH^{\bullet}$ (32%) and superoxide (7.57%) radicals, as well as to reduce the radical Fe^{3+} [80]. Continuing with muscle hydrolysates, Pei-Teng et al [81] studied the enzymatic hydrolysis of TGGG grouper

186 fillets using four different enzymes (Alcalase, proteinase K, trypsin and pepsin). The authors
187 report that the hydrolysates obtained with pepsin exhibited the highest reducing power (30%
188 against the Fe^{3+} ion [81]).

189 Waste from the aquaculture industry has also been the object of study to obtain
190 hydrolysates, as shown by the work of Tejpal et al [62], who evaluated the effect of keeping
191 a mixture of tilapia waste in refrigeration on the biofunctional properties of the hydrolysates
192 obtained after processing with pepsin. The results of this study suggested that although the
193 refrigeration process has a negative effect on the antioxidant activity of the hydrolysates, they
194 have the same or greater capacity to reduce Fe^{3+} than synthetic antioxidants such as BHA
195 and BHT [62].

196 Recent investigations have shown that pepsin can work adequately in tandem with
197 other proteases as it happens in the digestive tract. One of the most studied systems is the
198 pepsin-trypsin system, which has been used in the production of antioxidant peptides from
199 *Katsuwonus pelamis* (skipjack tuna) by-products (scales, heads and bones) [82–84],
200 *Pseudosciaena polyactis* (redlip croaker) scales collagen [85], *Lophius litulon* (monkfish)
201 muscle [86], and *Scomberomorous niphonius* (Spanish mackerel) muscle [87]. From skipjack
202 tuna bones, it was possible to identify the sequence of a peptide fraction (Gly-Ala-Glu-Gly-
203 Gly- Ile-Gly) that, at a concentration of less than 0.5 mg/mL, presented half of the maximum
204 effective concentration to inhibit the radicals DPPH, hydroxyl radical, superoxide anion
205 radical and ABTS cation radical [82]. For its part, from the flake gelatin of the skipjack tuna
206 hydrolysate, the peptide Asp-Gly-Pro-Lys-Gly-His exhibited a high radical scavenging
207 capacity with EC50 values of 0.54, 0.41 and 0.71 mg/mL for DPPH radicals, hydroxyl and
208 superoxide anion, respectively [84], which are higher values than those reported in extracts

209 of vegetable raw materials such as barley seeds (*Hordeum vulgare L.*) [88] . Finally, from
210 skipjack tuna head hydrolysates, a peptide fraction whose maximum effective concentration
211 (EC50) against DPPH radical, hydroxyl radical and superoxide anion radical was less than
212 0.6 mg/mL, was identified as Trp-Met-Phe-Asp-Trp [83]. In the case of the Monkfish muscle
213 hydrolysates and redlip croaker scales collagen, two peptides were identified, Tyr-Trp-Asp-
214 Ala-Trp and Glu-Gly-Pro-Phe-Gly-Pro-Glu-Gly, respectively. These have great potential to
215 be used in the treatment of liver diseases associated with oxidative stress, since these peptides
216 had the capacity to promote the activation of intracellular enzymes related to oxidative
217 balance such as superoxide dismutase, catalase and glutathione peroxidase, as an additional
218 property to the antioxidant activity presented by Monkfish muscle hydrolysate and redlip
219 croaker scales peptide against the DPPH radical (EC50 0.51 and 0.37 mg/mL, respectively),
220 to the hydroxyl radical (EC50 0.32 and 0.33 mg/mL, respectively) and the superoxide anion
221 radical (EC50 0.48 and 0.47 mg/mL), respectively [85,86]. In the same way, as in the studies
222 presented above, by means of the hydrolysis of the Spanish mackerel muscle, the
223 identification of a highly antioxidant peptide (Gly-Tyr-Asp-Trp-Trp) was achieved whose
224 capacity to stabilize DPPH$^{\bullet}$, superoxide (O_2^-) and hydroxyl (OH^-) radicals was greater than
225 80% using concentrations below 2 mg/mL. The production of fish hydrolysates using
226 enzymatic systems has also shown good results when mixing pepsin with pancreatin, the
227 foregoing is evidenced in the work of Chel-Guerrero et al [89], who managed to obtain a
228 peptide from *Pterois volitans* L. (lionfish) muscle with the ability to stabilize DPPH$^{\bullet}$
229 (54.27% radical decrease) and ABTS$^{\bullet}$ (107.31 TEAC mM/mg protein) [89].

230 Obtaining protein hydrolysates has not only been carried out on raw materials with a
231 high protein content, studies have also been carried out on specific protein groups, such as

232 sarcoplasmic, myofibrillar [90], collagen [91] or gelatin [92]. Shiao et al [93] studied the
233 production of antioxidant peptides from tilapia flake gelatin, hydrolyzed with the pepsin-
234 pancreatin system to simulate the digestive process. The authors report the presence of two
235 peptides (Gly-Tyr-Asp-Glu-Tyr and Glu-Pro-Gly-Lys-Ser-Gly-Glu-Gln-Gly-Ala-Pro-Gly-
236 Glu-Ala-Gly-Ala-Pro), which showed great capacity to stabilize the $ABTS^{\bullet}$ radical,
237 presenting values of Trolox equivalents higher than 20 μM when concentrations of 0.5
238 mg/mL were used [93].

239 As it can be seen throughout this section, the studies show the excellent antioxidant
240 activity of the peptides obtained with pepsin. However, it is important to note that the
241 analyses performed are *in vitro* studies, and the methods used for these studies are not very
242 specific and differ from biological systems [72]. For this reason, it is necessary for the
243 investigations carried out on the antioxidant activity of peptides (and in general of all
244 biological properties) to be directed to real *in vivo* tests in pre-clinical and clinical studies,
245 which generate scientific evidence of their true biological potential and thus allow have a
246 more precise estimation of the antioxidant power of these molecules in the organisms that
247 use them. This is crucial since even the safety of peptides becomes questionable, since most
248 toxicological research is carried out *in vitro* and on animals. Thus, to substantiate the safety
249 claimed for these compounds, the level of evidence supporting the safety of ingesting
250 bioactive peptides must be increased [94].

251 **Anti-inflammatory activity**

252 Inflammation, as part of the host defense mechanism against inflammatory inducers
253 (microbial infections, noxious chemical and mechanical agents, and conditions such as tissue
254 infection and injury), maintains tissue homeostasis under different noxious conditions, and

allows patient survival [95,96]. A controlled inflammatory response is beneficial; however, excessive or uncontrolled inflammation and oxidative stress are known to promote the onset of chronic human diseases such as diabetes, obesity, cardiovascular disease, cancer, respiratory disorders, atherosclerosis and neurodegenerative diseases [97,98]. For this reason, the use of synthetic anti-inflammatories has been necessary, which allows controlling the overproduction of inflammatory mediators and the overresponse of enzymes involved in the inflammatory process [99]; nevertheless, prolonged use of these medications has negative consequences for the organism [100]. That is why there is currently a growing interest in finding anti-inflammatory compounds of natural origin, which do not have secondary effects on the body. In this regard, it has been reported that proteins and peptides from plant and animal sources (soybean, milk, fish meat and eggs) [101] have a potent anti-inflammatory activity, being capable of reducing the risk of disease such as cardiovascular disease [102].

In this context, there are some reports in which peptides with significant anti-inflammatory activity have been obtained from fish protein using the enzyme pepsin, which are reviewed below.

An anti-inflammatory tripeptide was purified and identified from the protein hydrolysate obtained from salmon pectoral fin after pepsin hydrolysis. It was shown, in *in vitro* tests, that the tripeptide Pro-Ala-Tyr (with 349.15 Da) inhibited the production of prostaglandin E2 and nitric oxide by 45.33% and 63.80%, respectively. In addition, the tripeptide significantly suppressed the protein expression of inducible cyclooxygenase-2 and nitric oxide synthase, and attenuated the production of pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin-6 and -1β [103].

In another work, peptides produced by pepsin and trypsin in the stepwise digestion of salmon myofibrillar protein conjugated with alginate oligosaccharide were evaluated *in vitro* and *in vivo* (Murino´s model) systems. Salmon myofibrillar peptides significantly reduced the secretion of the proinflammatory mediators (tumor necrosis factor (TNF)-α, nitric oxide and interleukin (IL)-6) as well as mRNA expression of inducible nitric oxide synthase, cyclooxygenase-2, TNF-α and IL-6. Also, the obtained peptides inhibited acute inflammation in a carrageenan- induced model of paw edema in mice [104].

Catalyzed hydrolysis of soluble collagen was used to produce collagen peptides from *Chanos chanos* (milkfish) scales, which were evaluated *in vitro* in terms of antioxidant, anti-inflammatory, and DNA-protective activities. The obtained peptides possess both high antioxidant activities and anti-inflammatory properties by reducing nitric oxide radicals and lipoxygenase activity. Moreover, milkfish scales collagen peptides treatment can directly protect against cyclobutane di-pyrimidine production and DNA single-strand breaks [105].

Gao et al [101] carried out an *in vitro* study to determine the anti-inflammatory potential of peptides produced by pepsin hydrolysis of sturgeon in a lipopolysaccharide (LPS)-induced RAW264.7 inflammatory model. They reported that pepsin hydrolysates significantly reduced the inflammatory cytokines (IL-6, TNF-α and IL-1β) and inflammatory mediator (nitric oxide) expression in a dose-dependent manner. Moreover, it was found that a purified sturgeon peptide exerted anti-inflammatory influence by the inhibition of mitogen-activated protein kinases (MAPKs) pathways and nuclear factor-κB (NF-κB) [101].

Finally, Sugihara et al [106] reported an interesting and useful method for producing novel anti-inflammatory peptides derivatives from fish myofibrillar protein. In this study, chum salmon myofibrillar protein was digested by pepsin-trypsin and conjugated to alginate

oligosaccharide through the Maillard reaction [106]. The obtained alginate oligosaccharide-conjugated peptides, which were successfully recovered using isoelectric focusing without the use of carrier ampholytes (autofocusing), strongly suppressed, in an *in vitro* system, the production of inflammatory cytokines, and this anti-inflammatory effect was enhanced with increasing amounts of alginate oligosaccharide bound to digested myofibrillar protein through the Maillard reaction [106].

As observed in the previous section of antioxidant activity, the studies carried out on the anti-inflammatory activity of peptides obtained from fish residues by hydrolysis with pepsin are *in vitro* studies (except for the one reported by Saigusa et al. 2015 [104], which even though they show an excellent anti-inflammatory activity, do not guarantee that said activity is presented in the same way in an *in vivo* system, or even more, directly in the human being. In this way, and as it occurs with the other reported activities, it is clear that there is a great need to carry out the studies towards their evaluation in more complex systems (animal models) and later to be evaluated in humans, since only in this way, the doors will be opened for their intensive use as substitutes for conventional drugs against these conditions.

Angiotensin I-converting activity

A key risk factor for inducing cardiovascular disease is hypertension, a chronic disease that causes more than nine million deaths per year and affects an estimated one billion people [107]. In humans, blood pressure is regulated through the renin-angiotensin-aldosterone system through the action of two main proteases, renin and angiotensin-converting enzyme (ACE) [108]. Particularly, angiotensin-converting enzyme is a useful therapeutic target for the treatment of hypertension since this enzyme can convert angiotensin I to angiotensin II and increase blood pressure by vasoconstriction [109]. For this reason, the

drugs used to treat hypertension, such as fosinopril, enalapril, captopril, benazepril and lisinopril, are designed to inhibit the activity of the angiotensin-converting enzyme, thus reducing angiotensin II levels [110]. Although these synthetic drugs are effective in treating the disease, it is well documented that they often cause side effects such as erectile dysfunction, hypotension, taste disturbance, persistent dry cough, angioedema, skin rashes and congenital malformations [64,111–114]. This has generated the need to explore new drugs that are less harmful to the body, which has led to the discovery of an increasing number of natural compounds capable of inhibiting angiotensin-converting enzyme [115]. An example of such compounds are bioactive peptides [108], which have low or no toxicity or side effects [116], and are obtained from the hydrolysis of various proteins from products such as fishes [117], *Spirulina platensis* [118], corn gluten meal [119], alfalfa [120], cheese whey [121], or many others.

In the particular case of fish as a source of protein to obtain peptides by hydrolysis with pepsin, some studies have reported that such peptides have angiotensin I-converting enzyme inhibitory activity. These works are described below.

Khiari et al [122] produced bioactive peptides from fish skin. For this target, gelatin was extracted from *Scomber scombrus* (mackerel) skin and subjected to hydrolysis with pepsin for 1, 2, 6 and 24 h. As result, the hydrolysate obtained after 24 h of hydrolysis exhibited high ACE-inhibitory activity (78.1%) and was able to significantly inhibit platelet aggregation by about 30%, which corresponds to moderate antithrombotic activity [122].

In another study, anti-hypertensive peptides were purified from a hydrolysate of flounder fish muscle. Pepsin hydrolysate showed the strongest angiotensin-I converting enzyme inhibitory activity, and from this, two new peptides, MEVFVP (721.2 Da) and

346 VSQLTR (703.4 Da) with IC50 values of 79 μM and 105 μM, respectively were obtained.
347 The Lineweaver-Burk plots suggested these peptides act as a competitive and a non-
348 competitive inhibitors of ACE, respectively. In addition, the administration of MEVFVP and
349 VSQLTR (40 mg/kg) reduced systolic blood pressures in spontaneously hypertensive rats,
350 with maximal decrements of 44.25 and 34.25 mmHg, respectively, similar to the obtained by
351 captopril administration (39.75 mmHg) [123].

352 Later, angiotensin-converting enzyme inhibitory peptides from extracted tilapia skin
353 and hybrid catfish skin collagen using pepsin were studied. It was found that hybrid catfish
354 skin collagen hydrolysate prepared by pepsin showed the higher ACE inhibitory activity
355 when compared to the activity found in tilapia skin hydrolysates. Additionally, after cation
356 exchange and two steps of size exclusion chromatography, hybrid catfish skin peptides
357 showed ACE inhibitory activity of 72 % [124].

358 In another interesting work, in which different enzymes and different hydrolysis times
359 were evaluated in the preparation of hydrolysates from the head and bones of hybrid grouper
360 (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*), it was found that Alcalase was the
361 most effective enzyme in the hydrolysis and produced hydrolysates with the higher
362 antioxidant activities, but Proteinase K and pepsin hydrolysates at a longer hydrolysis time
363 resulted in a higher ACE-inhibitory activity [125]. This study shows that, even on the same
364 raw material, the use of different enzymes at different hydrolysis times can generate different
365 peptides with varied biological activity, with a greater or lesser degree of hydrolysis, so that,
366 through the exploration of different enzymes (with their respective operating conditions) it
367 is possible to select those that produce the peptides with the best biological activity(ies).

368 *Euthynnus affinis* (kawakawa) protein hydrolysate was produced by pepsin extracted
369 from skipjack tuna. Kawakawa protein hydrolysates were separated into four different
370 fractions, and the results indicated that the fractions showed angiotensin converting enzyme
371 inhibition with IC50 values ranging from 0.45 to 1.86 mg/mL with higher activity in the
372 fraction with molecular weight <1kDa [126].

373 Finally, bioactive peptides were produced from the fish *Gadidae* and beef skeletal
374 muscles by 8 h of pepsin hydrolysis. Fish peptide composed of 21 amino acid residues and
375 beef peptide composed of 34 amino acid residues displayed angiotensin-converting enzyme
376 inhibitory activity with a half maximal inhibitory concentration (IC50) values of 7.3 μg/mL
377 and 5.8 μg/mL, respectively [127].

378 In the studies presented here, it has been possible to obtain peptides with significant
379 angiotensin inhibitory activity, similar to and even higher than that reported for peptides
380 obtained from other raw materials and with other enzymes (for example, chickpea protein
381 peptides with IC50 values ranging from 0.101 to 37.33 μg/mL prepared using papain,
382 pancreatin or Alcalase [128], Alcalase casein hydrolysate with an angiotensin I- converting
383 enzyme inhibitory activity of 62.5% [129], and *Jatropha curcas* peptide with an IC50 value
384 of 4.78 g/mL obtained by Alcalase hydrolysis [130]. At this point, it is important to mention
385 that the strong activity found for the peptides obtained with pepsin can be attributed to the
386 pepsin specificity by the amino acid residues at position P1 and P1′ with a preferential
387 cleavage in hydrophobic residues, since it has been reported that hydrophobic amino acids
388 such Met, Val, Ala, and Tyr, increase the ACE inhibitory potential as they can bind to the
389 catalytic site of ACE [115].

On the other hand, we also want to mention that most of the reviewed works suggest that the peptides obtained can be used in the production of functional foods, nutraceuticals and pharmaceuticals; however, it is necessary to note that in order to achieve these objectives, it is necessary to delve deeply into the activities reported for these peptides. In this review we found that few works compare the activity of the peptides obtained with the activities presented by the drugs available in the market (enalapril, captopril, benazepril). This is important, since in order to extend the use of peptides to the pharmacological area, they must be competitive with commercial drugs, and this must be scientifically demonstrated. In addition to that, said comparison must be both *in vitro* and *in vivo*, since the legal requirements in this area request, in order to accept that the peptides enter to the market and can be consumed by people, that tests must be carried out in humans. In this sense, it is necessary to carry out more studies on the purification of specific bioactive peptides and determination of the sequence of ACE-inhibiting amino acids; studies confirming the positive bioactive properties of isolated peptides using synthetic peptide models and comparing them with existing drugs, and of course, conducting observational and intervention studies in humans.

Other biological activities

411 In addition to the intensively explored antioxidant, angiotensin I-converting enzyme
412 inhibitory and anti-inflammatory activities, other highly attractive activities have been
413 reported in fish protein hydrolysates obtained using pepsin hydrolysis, which we summarize
414 in this section.

415 Large-scale use of antibiotics has caused the current crisis of antibiotic resistance,
416 which is an emerging global health problem listed by the World Health Organization among
417 the top ten global public health threats facing humanity [131]. For this reason, there is
418 currently an urgent and growing need for the development of new antibiotics and antibiotic
419 substitutes [132]. In this context, antimicrobial peptides, which are produced from the
420 synthetic and natural sources, arise as an excellent candidate to overcome antibiotic
421 resistance [133,134]. These peptides possess different mechanism of actions, high specificity,
422 low toxicity, a broad-spectrum antimicrobial activity [135], and they can have a synergistic
423 effect when used with conventional antibiotics [136]. In this regard, Wald et al [137]
424 produced antimicrobial hydrolysates from trout by-product using trout pepsin. The
425 hydrolysates demonstrated inhibitory activity against several gram-negative and gram-
426 positive bacteria, mainly fish farming bacteria *Flavobacterium psychrophilum* and
427 *Renibacterium salmoninarum*. It was found that the degree of hydrolysis exerts a
428 considerable influence on antibacterial activity, and the highest antibacterial activity was
429 obtained at a degree of hydrolysis of 30% [137].

430 Another type of bioactive peptides of great importance are immunomodulatory
431 peptides, which act through stimulation or suppression to maintain a disease-free state in
432 normal or diseased people, thus supporting the immune system, which is our first and main
433 means of protection against disease [138,139]. In this sense, some studies carried out with

peptides or hydrolysates obtained from fish proteins by hydrolysis with pepsin have shown that such peptides are capable of improving the immune system. Chalamaiah et al [138] evaluated the immunomodulatory effects of protein hydrolysates prepared from underutilized *Labeo rohita* (rohu) egg (roe), by enzymatic hydrolysis using pepsin, trypsin and Alcalase, in BALB/c mice. Results showed that pepsin hydrolysate significantly increased the splenic NK cell cytotoxicity, macrophage phagocytosis and level of serum immunoglobulin A, and pepsin and Alcalase hydrolysates significantly enhanced the mucosal immunity in the gut. The results of this study suggested that rohu egg protein hydrolysates were able to modulate immune function maybe due to the presence of immunostimulatory peptides [138]. In another work, protein hydrolysates from underutilized common *Cyprinus carpio* (carp) egg were prepared by hydrolysis with pepsin, trypsin, and Alcalase. The carp egg protein hydrolysates were orally administered daily to female BALB/c mice during 45d, finding that the three hydrolysates significantly enhanced the proliferation of spleen lymphocytes. In addition, pepsin hydrolysate significantly increased the splenic natural killer cell cytotoxicity, mucosal immunity (secretory immunoglobulin A) in the gut and level of serum immunoglobulin A [140]. The results obtained in the aforementioned works suggest that the immunomodulatory peptides or hydrolysates obtained can be used in various applications in industries such as food, nutraceutical and pharmaceutical.

Of particular interest are the immunomodulatory peptides with anti-allergic potential, which are among the most promising treatment of IgE-mediated food allergic diseases [141]. Concerning that point, an anti-allergic peptide from *Salmo salar* (Atlantic salmon) byproduct contained in the hydrolysate produced by pepsin hydrolysis, was purified and identified as Thr-Pro-Glu-Val-His-Ile-Ala-Val-Asp-Lys-Phe which proved to exert anti-allergic activity

457 after synthesis by inhibiting the release of β-hexosaminidase in IgE-mediated RBL-2H3 cell
458 degranulation at IC50 value of 1.39 mg/mL [142]. According to these results, Atlantic salmon
459 by-product can be a potential source of novel peptides which can be used as ingredients in
460 pharmaceuticals and food for food allergy management [142].

461 On the other hand, in the search for alternatives that allow the control of obesity, a
462 disease that has reached epidemic levels and that promotes the appearance of other serious
463 metabolic disorders (hepatic steatosis, dyslipidemia, type 2 diabetes mellitus and insulin
464 resistance) [143,144], it has been reported that bioactive peptides have an important role
465 [145]. In this way, Mizushige et al [146], examined the effects of Alaska pollack protein
466 hydrolysate digested artificially with pepsin and pancreatin on white adipose tissue and
467 skeletal muscle, finding that, the Alaska pollack protein hydrolysate group showed
468 significantly lower weight of white adipose tissue and higher weight of soleus muscle, and
469 reduced food intake and mRNA expressions of neuropeptide Y and agouti-related protein in
470 the hypothalamus, compared with the control group. The authors conclude that the anti-
471 obesity activity of the hydrolysate is maybe due to the reduction of appetite and the
472 enhancement of basal energy expenditure by skeletal muscle hypertrophy in rats [146].

473 Finally, Yang et al [147] demonstrated, using *in vitro* simulated gastrointestinal
474 digestion with pepsin and in silico studies, that it is possible to obtain monoamine oxidase A
475 inhibitory peptides from *Trichiurus japonicus* (hairtail). Among the synthesized peptides,
476 Val-Val-Phe-Glu-Val-Phe-Trp showed the highest monoamine oxidase A inhibitory activity
477 (IC50 = 0.405 mM) [147]. Selective monoamine oxidase A inhibition increases the level of
478 serotonin in the central nervous system and thus reduces symptoms of clinical depression
479 [148]; in this sense, the hairtail monoamine oxidase A inhibitory peptides obtained can be

used as functional ingredients for monoamine oxidase A inhibition or potential alternatives for antidepressant [148].

Practical utilization of fish peptides

As it has been shown in this review and in multiple scientific works, peptides obtained from protein hydrolysis have a wide variety of biological properties, which has led to these biomolecules being used in different industrial processes. An example of the above is the approval by the FDA for use in preclinical studies of more than 60 antioxidant peptides. [149], the use of hydrolysates in animal feed [150], the incorporation in cosmetic products [151] and its implementation in different processes of the food industry [152]. In this sense, a series of works are presented below in which different uses of peptides obtained by proteolysis with pepsin of proteins of fish origin are highlighted.

One of the most studied uses of peptides has been their incorporation in the formulation of diets for the rearing of aquatic organisms, both to improve their growth, and in the preparation of functional foods that allow the prevention of diseases [153]. In the first case, the work of Srichanun et al [154] stands out. He managed to improve the digestive capacity, larval growth and survival of *Lates calcarifer* Bloch larvae by including up to 25% hydrolysates of fish muscle or squid mantle obtained with the pepsin-Alcalase system [154]. In aquaculture, hydrolysates have also been shown to improve the immune system of fish when they are fed with diets enriched with peptides. This was demonstrated by Luo et al [155], who supplemented the diet of *Larimichthys crocea* (yellow croaker) with muscle hydrolysates from *Michthys miiuy*, finding that a dose of 1.2 mg/fish of bitter peptides favors an increase in the activity of leukocytes and lysozyme compared to the control group [155].

The inclusion of peptides derived from fish in foods is not only due to the biological characteristics highly described in this review. It also responds to the fact that these protein fractions have different physical or chemical properties of great interest to the food industry [156]. In accordance with the above, a study was published in which hydrolysates of *Pangasianodon hypophthalmus* viscera obtained by enzymatic hydrolysis with pepsin were applied by spray-drying [157]. This hydrolysate presented excellent technological properties such as water retention capacity (0.84 ± 0.03 mL/g), oil absorption capacity (1.57 ± 0.04 mL/g), emulsion stability index (87.98 ± 2.13 min), which make it an interesting ingredient for incorporation in foods that need to improve their technological properties [157]. Following the application of peptides in the food industry, it was successfully demonstrated that these protein fractions inhibit the oxidation of cold-preserved shrimp myofibrillar proteins, providing these results with an alternative to increase the shelf life of highly perishable products [158].

Finally, it is important to mention that bioactive peptides have been explored beyond their biological properties, using them in the size regulation, stabilization, and functionalization-based surface modification during the synthesis of selenium nanoparticles which have a very important role in therapeutic applications [159]. Fish peptides obtained with pepsin have not been the exception, and it has been reported that they have been used in the development of a peptide-selenium complexes, taking advantage of the richness of the carboxyl and amino groups of the peptides obtained with pepsin, which interact with selenium, transforming the secondary protein from β sheet to α helix and β turn, obtaining a nanocrystalline structure, which can be employed as templates to stabilize selenium nanoparticles [160].

This section shows that, although interesting practical applications of bioactive peptides obtained from fish waste using pepsin have been explored, there are very few studies dedicated to investigating this topic. In addition, with respect to their application in humans, there is a great void to fill, since despite the fact that many studies describe the excellent biological properties that these peptides present, in this review we did not find any study that deals with the use of peptides in feeding or treatment of diseases in humans. This is a great limitation for the development of this field, since the ultimate goal of science is the application of the results for the benefit of humanity. As long as application studies are not carried out in human beings, it will be very difficult to extend the use of these compounds to the industry, and consequently, to society in general, since biological properties claims for bioactive peptides must be supported by substantial evidence from human studies [161].

Conclusion and futures perspectives

This review provides a comprehensive summary of recent research advances in the production of bioactive peptides derived from fish proteins by pepsin hydrolysis. Based on this, it is possible to state that fish protein derived peptides with various biological properties can be successfully produced by pepsin hydrolysis. However, it is important to note that there are several issues in this topic that require attention. For example, compared to enzymes such as Alcalase and papain, the studies carried out with pepsin are very scarce, which may be due to the high susceptibility of this enzyme with respect to the pH of the reaction, which can be an important limitation for its intensive use. In this sense, more studies should be carried out on the optimization of the operating conditions during the production of bioactive peptides or in the protection of this enzyme (i.e., enzyme immobilization), so that it could generate

549 the best results. In addition, although the use of pepsin is a central part of this work, it is
550 important to mention that, for an optimal use of fish residues, it is desirable and necessary to
551 explore other enzymes, such as papain, Alcalase, pancreatin, chymotrypsin, Flavourzyme,
552 Neutrase, etc., and their respective operating conditions (pH, temperature, time of hydrolysis,
553 etc.), since, as shown in this work, different enzymes generate peptides with different degrees
554 of hydrolysis and different biological properties.

555 Another very important point to note is that, most of the studies found focus on the
556 production, purification, identification and *in vitro* evaluation of bioactive peptides, and
557 suggest that they can be used in pharmacological or food products; however, there are still
558 very few studies on the application of these peptides, so it is necessary to scientifically
559 demonstrate that these bioactive peptides can be successfully used in the formulation of new
560 health-improving products; in this way, the migration from the laboratory to the industrial
561 application of these compounds will be promoted. In the same sense, before using bioactive
562 peptides at an industrial level, it is necessary to carry out studies in animals and humans, to
563 determine the final effects that the consumption of these products brings, to ensure that they
564 do not have secondary effects on the body, and that effectively, provide the properties that
565 have been demonstrated at the laboratory and in vitro level. In fact, in terms of bioactive
566 peptides, the lack of sufficient solid human data to support the health and safety claims of
567 such peptides is the main obstacle to the development of the bioactive peptide industry. It is
568 necessary to overcome this obstacle, and for that, research should be directed or focused on
569 generating information on mechanisms of action, interactions of other drugs or food
570 ingredients with bioactive peptides, safety, efficacy or which levels are beneficial for health,
571 absorption, distribution, bioavailability, metabolism, excretion, dose-response relation, and

even if they can be consumed with foods. These studies are necessary not only to show that the biological properties really provide a benefit to the human being, but they are also essential to comply with the legal regulations that some countries have decreed in this regard, which, although they have particularities, regulations such as the issued by Food and Drugs Act (Canada), Federal Food, Drug, and Cosmetic Act (USA), European Food Safety Authority (EU) and Japanese Ministry of Health, Labor, and Welfare (Japan), agree on the need to demonstrate the properties of peptides, carrying out intervention and observational studies in human. Only in this way will it be possible to fulfill the ultimate goal of obtaining compounds with beneficial biological properties for humans, which is precisely to make them accessible to humans through the development of new products that are produced on an industrial scale.

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Legend

Figure 1. Schematic representation of the production process of bioactive peptides from raw materials of fishery origin by enzymatic hydrolysis using pepsin.