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1 Assessment of the biological activity of fish muscle protein hydrolysates

2 using *in vitro* model systems

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10 Abstract

11 The generation of biologically active fish protein hydrolysates (FPH) is a useful technique to 12 produce value-added products with potential application in the functional food and 13 nutraceutical industries. Fish muscle is an attractive substrate for the production of protein 14 hydrolysates due to its rich protein content, containing 15-25% of total fish protein. This paper 15 reviews the production of protein hydrolysates from fish muscle, most commonly via 16 enzymatic hydrolysis, and their subsequent bioactivities including anti-obesity, 17 immunomodulatory, antioxidant, angiotensin I-converting enzyme (ACE)-inhibitory, anti-18 microbial, and anti-cancer activities as measured by in vitro testing methods. Disease 19 prevention with FPH potentially offers a safe and natural alternative to synthetic drugs. Small 20 molecular weight (MW) FPHs generally exhibit favourable bioactivity than large MW 21 fractions via enhanced absorption through the gastrointestinal tract. This review also discusses 22 the relationship between amino acid (AA) composition and AA sequence of FPH and peptides 23 and their exhibited in vitro bioactivity.

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25 Keywords: fish protein hydrolysates; enzymatic hydrolysis; in vitro; bioactivity

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Abbreviations: AA, amino acid; AAPH, 2,2-azobis-(2-amidino- propane) dihydrochloride: 27 28 ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; ACE, angiotensin-1-converting 29 enzyme; BMI, body mass index; BWPH; blue whiting protein hydrolysate; CAT, catalase; CCK, cholecystokinin; C/EBPa, CCATT/enhancer binding protein a; CFU, colony forming 30 31 unit, COX-2, cyclooxygenase-2; DH, degree of hydrolysis; DPPH, 2,2-diphenyl-1-32 picrylhydrazyl; EC₅₀, concentration corresponding to half-maximal activity; E/S ratio, 33 enzyme/ substrate ratio, FPH, fish protein hydrolysates; FOSHU, Foods for Specific Health 34 Use; FRAP, ferric reducing antioxidant power; GCB, graphitized carbon black; GI, 35 gastrointestinal; GLP-1, glucagon-like peptide-1; GSH, glutathione; GSH-Px, glutathione 36 peroxidase; GPH, goby protein hydrolysate; H₂O₂, hydrogen peroxide; IL, interleukin; LPH, 37 lanternfish protein hydrolysates; LPS, lipopolysaccharide; iNOS, inducible nitric oxide 38 synthase; MAPK, mitogen-activated protein kinase; MIC, minimum inhibition concentration; 39 MW, molecular weight; NF, nuclear factor; NO, nitric oxide; O₂⁻, superoxide anion; ORAC, 40 oxygen radical absorbance capacity; OH, hydroxyl; PPARy, peroxisome proliferator-activated 41 receptor γ; PGE₂, prostaglandin E₂; PYY, peptide YY; RPH, ray protein hydrolysate; ROS, 42 reactive oxygen species; RP-HPLC, reversed-phase high-performance liquid chromatography; 43 RSM, response surface methodology; SGID, simulated gastrointestinal digestion; SOD, 44 superoxide dismutase; SPH, sardinella protein hydrolysate; SREBP-1, sterol regulatory 45 element binding protein; TNF-a, tumour necrosis factor-a; ZPH, zebra blenny protein 46 hydrolysate.

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50 Fish is a rich source of protein, ranging from 10-25% depending on species, with 15-25% of 51 total protein located in fish muscle (Petricorena, 2015). Fish muscle proteins can be divided 52 into 3 groups, namely structural protein (approximately 70-80% of total protein content), 53 myofibrillar protein and sarcoplasmic protein. The three groups contain all essential amino acids (AA); specifically, Lys accounts for 8.8%, Trp 1.0%, His 2.0%, Phe 3.9%, Leu 8.4%, Ile 54 55 6.0%, Thr 4.6%, Met-Cys 4.0% and Val at 6.0% (Hayes & Flower, 2013). Marine organisms 56 are reported to produce a variety of potent bioactive compounds as they are forced to live in a 57 complex environment which is exposed to extreme conditions of salinity, pressure, temperature 58 and illumination (Hamed et al., 2015). Bioactive peptides purified from fish sources have 59 garnered considerable interest in recent times with potential applications in both food and 60 pharmaceutical industries. Bioactive fish protein hydrolysates (FPH) and peptides are desirable 61 functional food ingredients due to their natural availability, relatively low-cost extraction 62 methods and their ability to exert a beneficial effect on human health by exhibiting antioxidant, anti-inflammatory, anti-proliferative, anti-hypertensive, and cardio-protective bioactivities 63 64 (Suleria et al, 2016). The introduction of the 'landing obligation' policy by the European 65 Commission in 2019 has maximized protein harvest from low-value fish species, thereby 66 presenting a profitable source of bioactive peptides.

Bioactive peptides which are usually inactive in the parent protein molecules can be released via enzymatic hydrolysis, chemical hydrolysis or fermentation. These biofunctional peptides generally range in size from 2-20 AA residues. However, the molecular weight (MW) and size of the peptides and the AA composition and sequence of the peptide ultimately influences their bioactive properties. 72 In vitro testing is often utilized for preliminary research, prior to testing via in vivo 73 model systems, due to its cost-efficiency and ability to yield rapid and reproducible data. 74 Although non-cellular bioassays are used to investigate some bioactivities including 75 angiotensin-1-converting enzyme (ACE) inhibition and anti-microbial activity, cellular model 76 systems are useful for investigating various bioactivities as well as unravelling the biological 77 pathways activated upon contact of the bioactive compound with the target cells. Although in 78 vitro studies provide a controlled environment for experimentation, cellular bioassays involve 79 maintaining cells outside of the living organism; therefore, results must be interpreted carefully 80 due to the innate complexity of organ systems in vivo (Jain et al., 2018). Bioactive FPH for 81 oral consumption face the challenge of surviving the hydrolytic conditions of the GI tract, so 82 that absorption through the gut barrier and contact with target cells is achieved.

This paper will critically review current knowledge emerging from *in vitro* model systems on the bioactive potential of protein hydrolysates and peptides isolated from various fish muscle sources (Figure 1). We highlight limitations of studies, as well identify gaps in the existing knowledge of bioactive fish peptides which has enabled us to recommend future research opportunities.

88 2 **Production of FPH**

The bioactivity of food-derived protein hydrolysate ultimately depends on peptide and AA composition. However, the composition of the resulting fraction is highly influenced by the protein source, method of hydrolysis, hydrolysis conditions and degree of hydrolysis (DH). Protein hydrolysates can be produced via (a) enzymatic hydrolysis with proteases sourced from various commercial animal, microbial and plant sources, (b) fermentation with proteolytic microorganisms, or (c) chemical hydrolysis with either alkali or acid. Chemical hydrolysis is the least common method of hydrolysis due to non-specific cleavage of peptide bonds resulting

96 in high variability in hydrolysate bioactivity. It is also known to yield products with reduced 97 nutritional value due to destruction of Cys, Arg, Thr, Ser, and Lys residues (Nasri, 2017; Provansal et al., 1975). However, it does play a role in bioactive peptide release during 98 99 gastrointestinal (GI) digestion of dietary protein (Dallas et al., 2017). Fermentation with 100 proteolytic microorganisms utilises starter and non-starter cultures available commercially 101 within the fermented food sector. Although Bacillus species are most commonly used for 102 fermentation of FPHs (Godinho et al., 2016; Jemil et al., 2014), Bkhairia and collegaues (2016) 103 reported poor efficiency of proteases from Pseudomonas aeruginosa A2 on hydrolysis of 104 golden grey mullet protein. Fermentation is the cheapest proteolysis process; however, 105 enzymatic hydrolysis is the most common method for producing bioactive protein hydrolysates 106 as specific proteases and conditions can be selected to produce hydrolysates of desired size, 107 sequence and bioactivity (Bhandari et al., 2020). The various proteolytic enzymes and 108 hydrolysis conditions employed for generation of bioactive FPH are presented in Table 1.

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110 <u>2.1. Microbial enzymes</u>

111 Microbial enzymes commonly used for the release of bioactive protein hydrolysates 112 from fish include Flavourzyme® (Aspergillus orvzae), Alcalase® (Bacillus licheniformis), 113 Neutrase® (Bacillus amyloliquefaciens), and Protamex[®] (Bacillus licheniformis 114 and Bacillus amyloliquefaciens). Fish muscle of Collichthys niveatus was rinsed, ground, 115 freeze-dried and sieved through a 120-mesh screen (125 micron) prior to hydrolysis with a 116 commercial microbial enzyme, either Flavourzyme®, Alcalase®, Neutrase® or Protamex® at 117 the same activity levels (10.103 U). Alcalase® hydrolysis induced the highest DH (17.03%) 118 compared with Neutrase® (15.04%), Protamex® (12.98%) and Flavourzyme® (5.82%) (Table 119 1) (Shen et al., 2012). DH is defined as the percentage of the number of peptide bonds cleaved 120 divided by the total number of peptide bonds in a protein. Seniman et al. (2014) also reported 121 catfish (*C.batrachus*) protein to be more susceptible to hydrolysis with Alcalase® than plant122 derived enzyme papain, demonstrating a direct correlation between DH and peptide content
123 (Table 1). In contrast, Fonseca et al. (2016) reported Cobia (*Rachycentron canadum*) meat
124 protein showed greater susceptibility to Protamex® hydrolysis exhibiting a DH value of
125 25.94% after 760 min compared to Alcalase® (10% after 300 min) and Flavourzyme® (12%
126 after 420 min).

127 <u>2.2. Animal-derived enzymes</u>

128 Digestive enzymes from bovine and porcine GI tracts such as pepsin, trypsin, and 129 chymotrypsin are also commonly used for production of biologically active protein 130 hydrolysates from various fish sources (Bkhairia et al. 2016; Chi et al. 2014; Darewics et al., 131 2014; Jiang et al., 2014; Kim & Byun, 2012; Ko et al., 2013; Naqash & Nazeer, 2010; Sung et al., 2012). DH values of bioactive hydrolysates were not always reported. Peptic rainbow trout 132 133 muscle protein hydrolysate exhibited a higher DH value (49.12%) and subsequent ACE 134 inhibitory activity than hydrolysates prepared with trypsin (DH 30.52%), or α -chymotrypsin 135 (DH 28.75%). However, all rainbow trout muscle protein hydrolysates showed greater 136 susceptibility to digestive proteases than Alcalase®, Neutrase®, or papain (Kim & Byun, 137 2012). Chi et al. (2014) reported that the most influential hydrolysis parameters on DH of 138 trypsin-prepared monkfish protein hydrolysates were temperature, pH, enzyme/substrate (E/S) 139 ratio and time, respectively. The maximum DH $(19.83 \pm 0.82\%)$ of monkfish protein 140 hydrolysate was obtained when hydrolysis conditions for trypsin were 40°C, pH 8.0, E/S 2% 141 with 4 h hydrolysis period (Table 1). Under-utilised skipjack tuna (Katsuwonus pelamis) 142 protein was highly susceptible to hydrolysis with either trypsin or Protamex® exhibiting DH 143 values of 71.68% and 78.33%, respectively, however, trypsin was chosen for future hydrolysis 144 of skipjack tuna protein due to its more attractive price point (Liu et al., 2015). A study by Darewicz et al. (2014) compared *in vitro* and *ex vivo* hydrolysis of salmon myofibrillar and sarcoplasmic proteins and reported *in vitro* hydrolysis with porcine pepsin or pepsin and Corolase PP was more efficient than human gastric or gastric and duodenal juices, respectively, as less intact protein was measured in *in vitro* hydrolysed fractions, indicating further hydrolysis and production of small MW peptides. This study demonstrates the complexity of the human digestive system and confirms that *in vitro* results may not always predict *in vivo* results.

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153 <u>2.3. Plant-derived enzymes</u>

154 Some studies found plant-derived proteases induced a higher DH in FPHs than 155 alternatively sourced proteolytic enzymes. Catfish (Pangasius hypothalamus) meat protein 156 hydrolysed with papain and bromelain exhibited DH values of 31.16% and 29.36%, respectively, compared to a DH of 13.3% for the hydrolysate produced with the microbial 157 158 enzyme Neutrase® (Table 1) (Ha et al., 2017). Hydrolysis with bromelain increased the free 159 AA content most effectively from 28.00 g/kg protein to 58.02 g/kg protein compared with 160 papain and Neutrase® hydrolysis, however, bromelain hydrolysis had no effect on the 161 antioxidant activity of catfish protein. Both papain and Neutrase® hydrolysates demonstrated 162 increased radical scavenging activity compared with the non-hydrolysed control, potentially 163 owing to the reduced hydrolysis of bioactive peptides.

164

165 <u>2.3. Production challenges</u>

The shortcomings associated with enzymatic hydrolysis of food proteins include solubility and bitterness, both of which are highly influenced by DH, proteolytic enzyme and substrate employed. Bitterness and solubility issues impose sensory and processing challenges, respectively, and must be managed for the practical application of protein hydrolysates. 170 Hydrolysis of Collichthys niveatus protein with Neutrase® generated a hydrolysate with a 171 higher content of sweet and umami taste AAs (116.07 µg/mL) namely Ala, Asp, and Glu and less hydrophobic, bitter AA, Phe, than Alcalase® hydrolysis (Shen et al., 2012). Hydrophobic 172 173 peptides are associated with bitter taste due to the presence of two functional units, the binding 174 unit and the stimulating unit, responsible for binding with the bitter taste receptor and 175 determining site for bitterness, respectively (Ishibashi et al., 1988). Dauksas and colleagues (2004) reported that hydrolysates obtained by use of Alcalase[®] were more bitter than 176 hydrolysates obtained using Flavourzyme[®] as measured by sensory analysis. However, a 177 secondary treatment with Flavourzyme[®] did not further reduce bitterness of the fraction, 178 179 whereas, treatment of the FPH with n-butanol and cholestyramine resin did indeed reduce 180 bitterness via extraction of bitter bile compounds (Dauksas et al., 2004).

181 Although it is reported that a high DH is related to improved solubility due to changes in MW, hydrophobicity and polar groups (Leni et al., 2020), Liu et al. (2015) reported 182 183 hydrolysis of under-utilised skipjack tuna protein for 5 h completely degraded small MW peptides to AA and the highest soluble protein content (80%) was observed after 2.5 hr 184 hydrolysis with trypsin or Protamex. Glycation of the trypsin hydrolysate with alginate 185 186 significantly improved solubility (p<0.05) compared with the non-glycated fraction in the pH 187 range of 2-10 possibly due to the glycation-induced shift of isoelectric point towards a more 188 acidic pH. It is well known that hydrolysates generally show low solubility at their isoelectric 189 points, therefore it is probable that the basic pH of the trypsin hydrolysate influenced its 190 solubility.

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Biological activity of fish muscle protein hydrolysates and peptides as reported *in vitro* model systems

- **195 3.1 Inflammation modulatory activity**
- 196

197 Table 2 details studies investigating the ability of fish muscle hydrolysates to modulate signals 198 within inflammatory response pathways. The human body initiates inflammation in response 199 to various stimuli including infections, injury, and toxins in an attempt to heal itself. Activation 200 of macrophages is essential for initiation and continuation of defensive reactions as 201 macrophages release various pro-inflammatory cytokines such as tumour necrosis factor-a 202 (TNF- α), interleukin (IL)-6 and IL-1 β and inflammatory mediators such as prostaglandin E₂ 203 (PGE₂) and nitric oxide (NO) which improve tissue repair (Je & Kim, 2012). However, 204 prolonged or excessive inflammation is associated with a wide range of diseases, including 205 chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, 206 and cancer.

207 Kangsanant et al. (2015) identified a novel anti-inflammatory peptide from 208 Flavourzyme® hydrolysed tilapia muscle protein via gel filtration chromatography and 209 reverse-phase high-performance liquid chromatography (RP-HPLC) with an AA sequence of AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK 210 (MW: 211 6309.49 Da) (Table 2). The peptide (20 mg protein/mL) reduced NO production by $40.9 \pm$ 212 0.2% in the murine macrophage cell line RAW264.7 stimulated with lipopolysaccharide (LPS) 213 for 48h; which was 100 fold higher than that of the crude hydrolysate. The presence of 214 hydrophobic AAs residues in the purified peptide, namely, Ala, Phe, Leu, Val and Ile was 215 hypothesized to play a significant role in its NO-inhibitory activity. Although further 216 explanation regarding the relationship between hydrophobic AAs and their function in 217 inflammatory modulation is required, it has been reported that the presence of hydrophobic 218 AAs enhance attraction and reactivity with the cell membrane and possibly promote 219 downstream signalling pathways with anti-inflammatory effects (Sangtanoo et al., 2020). In a 220 previous study, Kangsanant et al. (2014) demonstrated that ultrasonic pre-treatment of tilapia 221 protein hydrolysate prior to Flavourzyme® hydrolysis demonstrated superior NO inhibitory 222 activity than non- pre-treated hydrolysates (p<0.05). It was suggested that ultrasonic pre-223 treatment promoted protein unfolding, thereby increasing enzyme accessibility to its cleavage 224 sites. Anti-inflammatory protein hydrolysates purified from Argentine croaker were reported 225 to be rich in AAs Glu, Asp, Lys, Leu, Arg, and Ala (Da Rocha et al., 2018). These hydrolysates 226 were produced from Argentine croaker isolate and Argentine croaker myofibrillar protein with 227 varying DH (DH: 10-20%) using either Alcalase® or Protamex® (Table 2). Interestingly, as 228 DH increased from 10 to 20%, MW distribution decreased and the content of hydrophobic AAs 229 increased (p<0.05). The hydrolysate produced from Argentine croaker myofibrillar via 230 Protamex® hydrolysis with DH 10% at 5.0 mg/mL exhibited greater NO inhibitory activity in 231 LPS- activated RAW264.7 cells (24h incubation) than all other hydrolysates tested (p<0.05) 232 suggesting a role for peptides in its bioactivity rather than just free AA content. Hydrolysates 233 prepared from Argentine croaker isolate and myofibrillar also demonstrated in vitro antioxidant 234 activity and microbial-inhibitory activity in Brochothrix thermosphacta, Listeria innocua, and 235 Staphylococcus aureus.

However, the ability of FPHs to regulate NO should not be used as the only indicator
of anti-inflammatory activity. The effects of hydrolysates on cytokine and immunoglobulin
levels should be included to shed light on the specific biochemical interaction through which
the hydrolysate induced its immunomodulatory effect. Sturgeon protein-derived peptides LysIle-Trp-His-His-Thr-Phe, Val-His-Tyr-Ala-Thr-Val-Asp-Tyr, and His-Leu-Asp-Asp-Ala-LeuArg-Gly-Gln-Glu which reduced NO concentration in LPS-stimulated RAW264.7 cells
(p<0.05), also inhibited the production of cytokine IL-1β at all concentrations tested (12.5-50.0

243 µM) (Gao et al., 2020). Treatment of LPS-induced RAW264.7 cells with peptides Val-His-244 Tyr-Ala-Thr-Val-Asp-Tyr (25.0 µM and 50.0 µM), and His-Leu-Asp-Asp-Ala-Leu-Arg-Gly-245 Gln-Glu (12.5 μ M and 25 μ M) also inhibited generation of IL-6 (p<0.05). Further investigation 246 revealed that it is probable that these peptides induced anti-inflammatory activity via 247 suppression of the mitogen-activated protein kinase (MAPK) signalling pathway through 248 down-regulation of phosphorylation of the biomarkers JNK and p38. The activation of 249 inflammatory factors is closely associated with the generation of intracellular reactive oxygen 250 species (ROS). Interestingly, sturgeon peptides also increased antioxidant enzyme superoxide 251 dismutase (SOD) activity in LPS-stimulated RAW264.7 cells compared with the LPS control 252 (p<0.01).

253 Sweetfish protein hydrolysates (200 μ g/ mL) prepared with pepsin, trypsin, or α -254 chymotrypsin for 12h significantly reduced NO production in LPS-challenged RAW264.7 cells 255 after 24h exposure compared with the LPS-control (p<0.05) (Sung et al., 2012) (Table 2). 256 However, only trypsin and α -chymotrypsin hydrolysates successfully inhibited production of 257 pro-inflammatory cytokines TNF- α and IL-6, and inflammation mediator PGE₂. Both 258 hydrolysates effectively attenuated mRNA expression levels of inducible nitric oxide synthase 259 (iNOS) and cyclooxygenase-2 (COX-2) via downregulation of nuclear factor (NF)- $_kB$ 260 (p<0.05), thereby implicating the MAPK pathway. The α - chymotrypsin hydrolysate appeared 261 to suppress the phosphorylation signal from ERK-1/2, although no statistical analysis was 262 reported (Sung et al., 2012). Ko and Jeon (2015) also investigated the NO-inhibitory effect of 263 club tunicate (Styela clava) protein hydrolysates prepared with digestive proteases, however, 264 reported superior NO inhibiting activity for Protamex® hydrolysed club tunicate protein. The 265 study was expanded to include information on the anti-inflammatory potential of three individual fractions with various MWs; >10 kDa (SFTPH-I), 5-10 kD-0a (SFTPH-II) and <5 266 kDa (SFTPH-III). SFTPH-I (200 µg/ mL) significantly reduced production levels of 267

268 inflammation mediators NO and PGE₂ (p<0.05) and pro-inflammatory cytokines IL-6, IL-1 β 269 and TNF- α (p<0.01) after 24h exposure to LPS-challenged RAW264.7 compared with 270 macrophage exposed to LPS alone. It was determined that SFTPH-I inhibited production of 271 pro-inflammatory mediators via reducing protein expression levels of iNOS and COX-2 and 272 attenuating phosphorylation of MAPKs (ERK, p38 and JNK) in activated macrophages.

273 FPHs have also demonstrated pro-inflammatory activity in vitro. Activation of pro-274 inflammatory cytokines can enhance host defence against infection in immunodeficiency 275 patients. Tilapia mince protein hydrolysate (100 and 800 µg/mL) produced by purified enzyme from *V.halodenitificans* SKI-3-7 significantly increased gene expression of IL-1β and COX-2 276 277 in the human monocyte leukaemia cell line THP-1 stimulated with LPS, after 6h incubation 278 (p<0.05) (Toopcham et al., 2017) (Table 2). Similarly, a low MW fraction (<1 kDa), labelled 279 NJP, isolated from papain hydrolysed Nibea Japonica protein (200 µg/ mL) significantly 280 upregulated protein expression of iNOS and production of NO in LPS-activated RAW264.7 281 cells (p<0.01) (Zhang, Hu, et al., 2019). At this concentration, it also increased production of 282 pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β and activated the NF- κ B signalling 283 pathway by upregulating NF- κ B pathway-related proteins including I κ B kinase (Ikk)- α and 284 IKK-β. The neutral red internalization model was employed to demonstrate that NJP increased 285 phagocytosis rate in RAW264.7 cells in a concentration-dependent manner; this initiated the 286 innate immune response via clearance of apoptotic cells or cellular debris.

Although the majority of *in vitro* immunomodulatory studies focus on regulation of pro-inflammatory cytokines IL-6 and TNF- α in LPS-stimulated macrophage, additional possible mechanisms are elucidated in *in vivo* studies, including the promotion of natural killer cells, stimulation of lymphocytes such as T cells and B cells, and stimulation of secretory immunoglobulin A (S-IgA), thereby enhancing levels of mucosal immunity in the gut. Additional clinical trials are necessary to understand the true effect of specific FPHs on the immune system. A study on the immunomodulatory potential of FPHs in humans reported salmon protein hydrolysate (Amizate) orally administrated (3 or 6g/ day, 4 months) to malnourished Indian school children induced no effect on serum immunoglobulins IgG, IgM or IgA or CD4/ CD8 lymphocyte ratio (Nesse et al., 2011).

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- 298 **3.2** ACE inhibitory activity
- 299

300 ACE inhibition by fish-derived protein hydrolysates represents a safer alternative than 301 synthetic hypertensive drugs (i.e., captopril; IC₅₀ value 0.004 mg/mL) for the therapeutic 302 management and treatment of cardiovascular diseases such as atherosclerosis, myocardial 303 infarction, and stroke. Suetsuna and Osajima (1986) were the first to confirm the presence of 304 ACE inhibitory protein hydrolysates in fish which they purified from sardine and hairtail 305 muscle via enzymatic hydrolysis with denazyme (Table 3). Wijesekara and colleagues (2011) 306 compared the effect of various proteases on ACE-inhibitory activity of hydrolyzed seaweed 307 pipefish muscle protein and reported Alcalase® hydrolyzed seaweed pipefish muscle 308 hydrolysates induced the greatest inhibitory effect on ACE followed by trypsin, papain, pepsin, 309 Neutrase® and pronase (Wijesekara et al., 2011). Peptides Thr-Phe-Pro-His-Gly-Pro (MW: 310 744 Da) and His-Trp-Thr-Gln-Arg (MW: 917 Da) subsequently purified from the Alcalase® 311 hydrolysate via chromatographic methods exhibited IC₅₀ values of 0.62 and 1.44 mg/mL, 312 respectively (Table 3). A study by Jiang et al. (2019) offered insight into the molecular binding 313 of ACE-inhibitory seaweed pipefish peptides to ACE protein. Both peptides effectively 314 interacted with ACE through hydrogen bonding and hydrophobic interactions with AAs at the 315 active site of ACE, thereby inhibiting the catalytic activity of ACE. The authors proposed that 316 the superior ACE-inhibitory activity of peptide Thr-Phe-Pro-His-Gly-Pro over His-Trp-Thr-317 Gln-Arg, owed to the formation of hydrophobic interactions with key ACE AAs Glu384 and

318 Glu41. Additionally, the ACE-Thr-Phe-Pro-His-Gly-Pro complex showed favourable total 319 binding energy (-167.599±49.637 kJ/mol), as well as van der Waals and electrostatic energy 320 than the ACE-His-Trp-Thr-Gln-Arg complex (total binding energy (-141.342±41.245 kJ/mol), 321 indicating formation of stronger complexes with ACE. The peptides are also reported to act via 322 non-competitive inhibition, therefore both peptide and substrate can both be bound to the 323 enzyme at any given time, forming enzyme-substrate-inhibitor and enzyme-inhibitor 324 complexes to inhibit ACE activity. Other fish sources of non-competitive ACE inhibitors 325 include seaweed pipefish, bigeye tuna, upstream chum salmon (Balti et al., 2015; Qian et al., 326 2007; Ono et al., 2006).

327 Peptic rainbow trout muscle hydrolysate exhibited superior ACE inhibitory activity 328 (IC₅₀ value of 0.61 mg/mL) compared with hydrolysates prepared with trypsin (IC₅₀ value of 329 1.09 mg/mL) and α -chymotrypsin (IC₅₀ value of 1.51 mg/mL) (Kim & Byun, 2012) (Table 3). 330 Fraction A with AA sequence Lys-Val-Asn-Gly-Pro-Ala-Met-Ser-Pro-Asn-Ala-Asn (1220 331 Da) purified from the peptic hydrolysate inhibited ACE activity by 50% at a concentration of 332 63.9μ M. However, this peptide was demonstrated via Lineweaver-Burk plots to act as a 333 competitive inhibitor against ACE, i.e., competed with substrate Hippuryl-Histidyl-Leucine for 334 the binding sites of ACE. Competitive ACE inhibitory peptides were also found in grass carp, 335 snakehead fish and upstream chum salmon (Chen et al., 2012; Ghassem et al., 2014; Ono et 336 al., 2006; Samaranayaka et al., 2010). Nakajima et al. (2009) also employed digestive proteases 337 for the hydrolysis of FPHs and compared the ACE-inhibitory activities of resulting fractions. 338 Atlantic salmon and Coho salmon hydrolysed with thermolysin demonstrated enhanced ACE 339 inhibitory activity (IC₅₀ values of 47.3 and 86.6 µg protein/mL, respectively) than pepsin plus 340 pancreatin hydrolysates (IC₅₀ values of 791 and 466 µg protein/mL, respectively) (Nakajima 341 et al., 2009). Subsequent ultrafiltration of the thermolysin hydrolysates followed by size 342 exclusion chromatograms deemed 380-920 Da fractions responsible for exhibited ACE-

inhibitory activity, possibly due to the presence of ACE inhibiting di- to penta- peptides richin AAs Ala, Met, Leu, Tyr, Phe and Trp.

345 In general, low MW fractions of FPHs demonstrate superior ACE-inhibitory activity 346 than large MW fractions. Goby muscle protein hydrolysate produced with crude protease 347 extract from smooth hound intestines demonstrated increased ACE-inhibitory activity with 348 increasing DH (p<0.05) due to the generation of small MW peptides (Nasri et al., 2014). Furthermore, fractionation of ACE-inhibiting Pacific hake FPH prepared via autolysis $(10^7 K)$. 349 350 paniformis spores/g fish mince) generated a low MW fraction (1-3 kDa) which exhibited 351 superior ACE inhibition $(66.91 \pm 4.38\%$ at 0.286 mg/mL) than the intact hydrolysate 352 $(55.06 \pm 0.66\%$ at the same concentration) (Samaranayaka et al., 2010) (Table 3). Most 353 inhibitory peptides in the fraction were reported to be short-chained, polar and containing few 354 hydrophobic AAs in their sequence. Chen et al. (2012) observed similar results with the <3355 kDa fraction of Alcalase® grass carp meat hydrolysate inducing the greatest inhibitory effect 356 on ACE and the >10 kDa fraction showing the lowest anti-ACE activity. A single tripeptide 357 Val-Ala-Pro purified from the <3 kDa fraction was subsequently observed to exhibit 358 remarkable ACE inhibitory activity inducing 50% inhibition at 0.00534 ± 0.00003 mg/mL. 359 Interestingly, the tripeptide Val-Ala-Pro was also purified from an enzymatic hydrolysate of 360 bovine casein (IC₅₀ value of 2.0 µM) (Maruyama et al., 1987). Various ACE inhibitory di- and 361 tripeptides were also purified from salmon muscle tissue prepared with pepsin and Corolase 362 PP and fermented (Bacillus sp. SM98011) shark meat protein hydrolysates (Darewicz et al., 363 2014; Wu et al., 2008) (Table 3). Although the 3-5 kDa fraction purified from red lionfish 364 protein exhibited the highest ACE inhibitory activity (43.57%) of the five fractions tested (>10, 365 5-10, 3-5, 1-3, <1 kDa), it was proposed that the superior anti-ACE activity of the 3-5 kDa 366 fraction was related to a higher content of hydrophobic AAs (40.33%) compared to the other 367 fractions (Chel-Guerrero et al., 2020). Indeed, several of these peptides have been shown to

survive gut transit via simulated gastrointestinal digestion (SGID) suggesting ACE-inhibiting
ability will be maintained when administered orally (Balti et al., 2015; Chen et al., 2012;
Elavarasan et al., 2016; Ghassem et al., 2014).

371 It is well known that not only peptide size and chain length influences ACE- inhibitory 372 activity, but also type and order of AAs in the sequence. Potent anti-ACE peptides produced 373 from cuttlefish (Sepia officinalis) muscle proteins via hydrolysis using crude enzymes from B. 374 mojavensis A2 and cuttlefish hepatopancreas, gel filtration chromatography and RP- HPLC 375 were identified as Val-Glu-Leu-Tyr-Pro, Ala-Phe-Val-Gly-Tyr-Val-Leu-Pro and Glu-Lys-Ser-376 Tyr-Glu-Leu-Pro via tandem mass spectrometry with corresponding IC₅₀ values of 5.22, 18.02 377 and 14.41 µM, respectively (Balti et al., 2015) (Table 3). It was proposed that the presence of 378 hydrophobic AAs and Pro at the C-terminal may influence the ACE inhibitory activity of the 379 peptide. ACE-inhibitory peptides containing Pro at the C-terminal were also observed in 380 seaweed pipefish muscle hydrolysates (Wijesekara et al., 2011) and Alcalase® protein 381 hydrolysates from snakehead fish sarcoplasmic extract (Ghassem et al., 2014). Gómez-Ruiz 382 and colleagues (2006) reported that the rigid structure of Pro can maintain the conformation of 383 the carboxyl group at the C-terminal in a way that favours ACE-inhibitory activity. Peptides 384 rich in Pro are also resistant to GI digestion increasing the likelihood of efficacy in vivo 385 (Segura-Campos et al., 2011).

The position of Trp residues is also important for ACE-inhibition and inhibition mechanism of the peptides. Peptides with Trp at the C-terminal residue, namely Ala-Trp, Val-Trp, Met-Trp, Ile-Trp, Leu-Trp with IC₅₀ values of 6.4, 2.5, 9.8 and 17.4 μ M, respectively, showed non-competitive inhibition (Table 3). Whereas, reversed sequence peptides with Trp at the N terminal including Trp-Ala, Trp-Leu, Trp-Met showed reduced ACE inhibitory activity and acted via competitive inhibition (Ono et al., 2006). Similarly, Phe-Leu showed non-competitive ACE inhibitory activity with an IC₅₀ value of 13.6 μ M. However, Leu-Phe 393 showed competitive ACE inhibitory activity with an IC₅₀ value of 383.3 μ M. A study by Enari 394 et al. (2008) purified 20 active di- and tripeptides from salmon muscle papain hydrolysate and 395 demonstrated the strongest ACE inhibition by Ile-Trp with an IC₅₀ value of 1.2 μ M.

396 Overall, the evidence for ACE-inhibiting peptides derived from fish muscle is strong 397 although the inhibitory mechanism is yet to be fully established owing to its complexity and/ 398 or multi-target nature (Manzanares et al., 2019). Although there is evidence of fish-derived 399 ACE-inhibiting peptides inducing anti-hypertensive effects *in vivo*, the majority of anti-400 hypertensive peptides are derived from animal products and plants (Lee & Hur, 2017).

- 401
- 402 **3.3** Antioxidant activity
- 403

404 Antioxidant FPHs serve as valuable ingredients in functional foods considering their ability to 405 extend shelf-life, as well as induce health benefits via promoting cellular redox balance. 406 Several studies reported the antioxidant activity of FPHs and their ability to modulate oxidative 407 stress pathways in vitro (Table 4). Oxidative stress is associated with many diseases including 408 cancer, diabetes, rheumatoid arthritis, chronic inflammation, and numerous neurodegenerative 409 diseases (Kumar et al., 2017). The antioxidant activity of marine hydrolysates and peptides has 410 mainly been assessed via scavenging activity of free radicals and ROS in non-cellular in vitro 411 assays, namely, 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-412 bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), oxygen radical absorbance capacity 413 (ORAC), hydroxyl (OH) radical scavenging activity and superoxide anion (O₂) radical scavenging activity. Other common non-cellular in vitro antioxidant assays include metal 414 415 chelating activity and ferric reducing antioxidant power (FRAP).

416 It is well known that protease and proteolysis conditions employed ultimately417 determines the size and AA composition of the resulting fraction, and thereby, plays a crucial

17

role in the generation of antioxidant hydrolysates. Bashir et al. (2018) prepared a number of 418 419 hydrolysates from red muscle and white muscle of Pacific chub mackerel (Scomber japonicus) 420 using varying proteolytic enzymes (Protamex® or Neutrase®) and observed significant 421 differences between antioxidant activities of resulting hydrolysates. Pacific chub mackerel red 422 muscle protein hydrolysed by Protamex® (50°C, 120 min), white muscle protein hydrolysed 423 by Protamex® (50°C, 120 min), and white muscle protein hydrolysed by Neutrase® (50°C, 30 424 min) exhibited the highest DPPH radical scavenging activity (71.69%), SOD-like activity 425 (32.84%) and ABTS radical scavenging activity (95.39%), respectively (Table 4). Optimum 426 hydrolysis conditions for generation of antioxidant protein hydrolysates from small spotted 427 catshark and stonefish were determined via response surface methodology (RSM), a statistical 428 method that optimises processes involving many variables (Vázquez et al., 2017; Auwal et al., 429 2017). Under optimal hydrolysis conditions predicted by RSM, bromelain stonefish protein 430 hydrolysates scavenged DPPH radical and chelated Fe^{2+} by 48.94% and 25.12%, respectively 431 (Auwal et al., 2017). However, radical scavenging activities were much lower for small spotted 432 catfish protein hydrolysates produced with Alcalase® (DPPH 12.4%, ABTS 5.1%) or esparase 433 (DPPH 16.0%, ABTS 7.3%) (Vázquez et al., 2017) (Table 4). Free AA compositional analysis, 434 MW size distribution, DH% or peptide sequence identification were not reported in these 435 studies.

Bkhairia et al. (2016) also investigated the effects of various proteases on antioxidant activity on golden grey mullet (*Liza aurata*) protein hydrolysates and expanded the study to include AA analysis of resulting fractions. Golden grey mullet hydrolysates were prepared with enzymatic preparations from *P. aeruginosa* A2 and *Bacillus subtilis* A26, crude enzyme from *L. aurata*, trypsin or esperase and subsequent antioxidant assays deemed the hydrolysate prepared with *B. subtilis* A26 proteases (rich in Glx, Gly, and Phe) exhibited the highest DPPH and ABTS radical scavenging activity (IC₅₀ values of 3.80 mg/mL and 0.47 mg/mL, 443 respectively) and the hydrolysate prepared with *P. aeruginosa* A2 proteases (rich in Arg) induced the highest reducing power as determined by its ability to react with potassium 444 445 ferricyanide and ultimately ferric chloride to form ferric-ferrous complex (absorbance of 446 1.061 ± 0.11 at 5 mg/mL). These hydrolysates (0.1 to 10 mg/mL) did not induce haemolysis of human erythrocytes, indicating their non-toxic effect and thereby, may be suitable for 447 448 nutraceutical application. Washing and membrane removal pre-treatments and ultrasonic 449 treatment of alkaline proteinase prepared grass carp hydrolysates did indeed improve 450 antioxidant capacity as measured by radical scavenging activities (DPPH and ABTS) as well 451 as FRAP, without diminishing its nutritive value (Zhang, Yang, et al., 2018). The removal of 452 oxidized compounds by washing and membrane removal pre-treatments also modified the 453 colour of the lyophilized hydrolysate from a slight yellowish to white, thereby broadening its 454 potential application in food.

455 Numerous studies have also reported the relationships between DH and antioxidant 456 activity of fish muscle hydrolysates. Although Li et al. (2012) and Klompong et al. (2007) 457 reported antioxidant hydrolysates prepared from grass carp protein and yellow stripe trevally to exhibit reduced radical scavenging activity and reducing power and higher Fe²⁺-chelating 458 459 activity with increasing DH, Rabiei et al. (2019) and Da Rocha et al. (2018) reported an 460 increase in radical scavenging activity of hydrolysates produced from Klunzinger's mullet and 461 Argentine croaker muscles with increasing DH. Alcalase® hydrolysed Argentine croaker 462 isolate (DH 20%) rich in aromatic AA Tyr and charged acidic AA Asp demonstrated higher 463 ABTS radical scavenging activity and metal chelating activity than Protamex® Argentine 464 croaker isolate and Argentine croaker myofibrillar protein hydrolysates produced with Alcalase® or Protamex® (p<0.05). Protamex® Argentine croaker myofibrillar protein 465 466 hydrolysate (DH 20%) rich in the aromatic AA Phe exhibited the highest FRAP (p<0.05) (Da 467 Rocha et al., 2018) (Table 4). Aromatic AAs are reported to improve the radical scavenging
468 activity of peptides via hydrogen donation to electron-deficient radicals (Wang et al., 2014).

469 Relative to DH, low MW fractions generally exhibit enhanced antioxidant properties 470 than large MW fractions. Low MW fractions, fraction 2 (985-2379 Da) and fraction 3 (658-471 923 Da), prepared from Flavourzyme® freshwater carp Catla catla showed highest DPPH 472 scavenging activity and FRAP, respectively (p<0.05) (Elavarasan & Shamasundar, 2017). 473 Subsequent AA analysis identified fraction 2 to be rich in AAs Gly, Pro and Tyr with a final 474 ratio of total hydrophobic AAs to total AA content of 42.31:1 (Table 4). A high proportion of 475 hydrophobic AAs has been reported in peptides/hydrolysates with high antioxidant activity; 476 including monkfish pentapeptides Glu-Trp-Pro-Ala-Gln, Phe-Leu-His-Arg-Pro and Leu-Met-477 Gly-Gln-Trp (Chi et al., 2014), and peptides Gly-Ala-Ala, Gly-Phe-Val-Gly, Gly-Ile-Ile-Ser-478 His-Arg, Glu-Leu-Ile, and Lys-Phe-Pro-Glu purified from spotless smoothhound 479 (Mustelus griseus) muscle (Wang et al., 2014), as well as pentapeptides Phe-Trp-Lys-Val-Val 480 and Phe-Met-Pro-Leu-His isolated from papain hydrolysed miluy croaker muscle (He et al., 481 2019) (Table 4). AA analysis also determined antioxidant whitemouth croaker muscle 482 hydrolysates to be rich in hydrophobic AAs Ala, Pro, Tyr, Val, Met, Ile, Leu and Phe (Lima 483 et al., 2019). Hydrophobic AAs are well known to act as protein donors or electron/lipid radical 484 scavengers.

Low MW fractions from round scad protein hydrolysate (<5 kDa) and cod protein hydrolysate (<3 kDa) exhibited higher radical scavenging activity and reducing power than larger fractions (>10 kDa and >5 kDa, respectively) (Jiang et al., 2014; Sabeena Farvin et al., 2014) (Table 4). Peptides His-Asp-His-Pro-Val-Cys and His-Glu-Lys-Val-Cys were purified from the <5 kDa fraction of round scad muscle protein hydrolysate and effectively scavenged DPPH radicals (EC₅₀ values of 0.068 ± 0.001 and 0.031 ± 0.001 mM, respectively) and O₂⁻ radicals (EC₅₀ values of 0.374 ± 0.002 and 0.382 ± 0.002 mM, respectively) (Jiang et al., 492 2014). The antioxidative activities of the peptides were proposed to be enhanced by the 493 participation of hydrophobic AAs and one or more residues of His, Pro and Cys. The imidazole 494 group and thiol group of His and Cys residues, respectively, promotes proton donation, thereby 495 stabilising ROS. In particular, Cys is one of 3 AA in glutathione (GSH), a potent endogenous 496 antioxidant in mammalian cells. Hydrolysates that are rich in Cys are likely to boost GSH 497 cellular pathways. It is possible that antioxidant activity not only depends on the presence of a 498 specific AA but also its quantity and position within the peptide sequence. The presence of His 499 in the centre of the His-Asp-His-Pro-Val-Cys sequence may have been responsible for its superior antioxidant activity over His-Glu-Lys-Val-Cys (Jiang et al., 2014). Low MW fractions 500 (5-10, 1-3, and <1 kDa) rich in His residues were also purified from Alcalase[®] hydrolysed red 501 lionfish protein and demonstrated high antioxidant activity, with copper-chelating activity of 502 503 approximately 88%, as well as inhibiting hydrophobic β -carotene discolouration by 80% 504 versus the negative control. His residues are indeed reported to have a strong binding affinity 505 for copper ions, thereby preventing copper toxicity (Chel-Guerrero et al., 2020).

Interestingly, goby FPH which exhibited significant antioxidant activity as measured by DPPH radical scavenging activity, lipid peroxidation inhibition, β -carotene bleaching inhibition, and metal chelating activity also effectively inhibited lipid peroxidation of turkey meat sausage by 50% by storage day 3 compared with the control (Nasri et al., 2013). As a result, it is possible that incorporation of goby protein hydrolysate as powder with turkey meat sausage may prevent oxidative deterioration and increase shelf life as a result.

512 FPHs have also been shown to enhance antioxidant defence systems in various cell 513 model systems. Protease N hydrolysed lanternfish protein hydrolysates (LPH) were 514 demonstrated to prevent hydrogen peroxide (H_2O_2)-induced oxidative cell damage in human 515 neuroblastoma cells (SHSY5Y) (Chai et al., 2013). MTT assay demonstrated that the viability 516 of cells exposed to H_2O_2 (400 μ M) increased (67.2-82.3%) in a concentration-dependent 517 manner upon addition of LPH (0.10-1.44 mg/mL) over 24h. DNA fragmentation of H₂O₂-518 treated SHSY5Y was also reduced dose-dependently when exposed to LPHs (0.37-0.73 mg/mL) for 24h as measured by agarose gel electrophoresis (Table 4). Tripeptide Phe-Tyr-Tyr 519 520 and dipeptide Asp-Trp were subsequently identified as the antioxidant peptide fractions from 521 LPH. The position of Tyr and Trp at the C-terminus was also reported in antioxidant tri-522 peptides derived from canola protein hydrolysate indicating Tyr and Trp positioning is 523 important for bioactivity (Cumby et al., 2008). Antioxidant peptides Val-Cys-Ser-Val and Cys-524 Ala-Ala-Pro purified from flounder fish muscle protein hydrolysates also demonstrated dose-525 dependent (12.5–100 µg/ mL) cytoprotective effects against 2,2-azobis-(2-amidino- propane) 526 dihydrochloride (AAPH)-induced oxidative stress in kidney epithelial Vero cells after 24h 527 incubation (Ko et al., 2013). Both peptides dose-dependently decreased DNA fragmentation 528 and total ROS (Table 4). Similarly, but at a higher concentration of 0.5 mg/mL, Alcalase® 529 hydrolysed European seabass protein hydrolysate reduced AAPH-induced oxidation in canine 530 kidney MDCK1 cells by $12.8 \pm 4.5\%$ compared with cells treated with AAPH alone (Altinelataman et al., 2019). In the same study, Alcalase® hydrolysed gilthead seabream 531 532 muscle hydrolysates also reduced AAPH-induced oxidation but only to 91.60% compared with 533 treated control (100%) albeit statistical analysis was not performed.

534 Large yellow croaker (*Pseudosciaena crocea*) protein hydrolysate (MW <3 kDa) which 535 effectively scavenged DPPH and O_2^- radicals, also regulated the antioxidant enzyme defence 536 system via dose-dependently (50-300 µg/mL) increasing levels of glutathione peroxidase 537 (GSH-Px), SOD and catalase (CAT) in H₂O₂-treated liver HepG2 cells (Zhang et al., 2016) 538 (Table 4). Antioxidant peptides Ser-Arg-Cys-His-Val and Pro-Glu-His-Trp were subsequently 539 isolated via ion exchange chromatography, gel chromatography and RP-HPLC. Peptides Glu-540 Asp-Ile-Val-Cys-Trp, Met-Glu-Pro-Val, Trp and Tyr-Trp-Asp-Ala-Trp (50 µM) isolated from 541 monkfish protein hydrolysate prepared via in vitro GI digestion with pepsin and trypsin 542 protected antioxidant enzymes SOD, CAT, and GSH-Px in in H₂O₂-stressed Hep-G2 cells (Hu 543 et al., 2020). Again, it is possible that the high content of hydrophobic AA and presence of Trp 544 at the C-terminal of peptide sequences played a role in the antioxidant capacities of these 545 peptides.

546 Of note is that immortalised cells are routinely used in these *in vitro* assays but may be 547 inherently oxidatively stressed due to their cancerous origins, compromising any antioxidant 548 readouts. Overall, it is evident that the antioxidant activity of fish muscle protein hydrolysates 549 is mostly studied via non-cellular in vitro testing, whereas studies which employed cellular 550 models are limited. Although numerous antioxidant fish muscle protein hydrolysates have been identified via in vitro testing, very few studies exist investigating the antioxidant activity of 551 552 fish muscle protein hydrolysates in vivo (Nazeer, Kumar, & Ganesh, 2012, Bashir et al., 2018). 553

- 554

Anti-microbial activity 3.4

555

556 Similar to antioxidant activity, this bioactivity widens the uses of hydrolysates beyond health 557 enhancement to extending shelf life of foods. Hydrolysis of fish proteins can produce various 558 small MW peptides some of which have been shown to exert remarkable antimicrobial activity 559 depending on their AA composition and structural characteristics. Most anti-microbial peptides 560 are amphipathic with a positively charged, hydrophobic face and thereby, defend against 561 bacterial activity directly via electrostatic interactions with the anionic bacterial membrane. 562 Anti-microbial peptides can exhibit pore-forming action in bacterial membrane evoking 563 leaking of intracellular contents or infiltrate the cell entrapped in macropinosomes which are 564 subsequently released into the host cytoplasm resulting in bacterial destruction (Valero et al., 565 2020). Peptide fractions prepared from yellowfin tuna muscle hydrolysed via SGID were 566 subsequently fractionated via either solid-phase extraction on C18 or graphitized carbon black 567 (GCB) sorbent for purification of medium-sized peptide and short-sized peptide fractions, 568 respectively (Cerrato et al., 2020). The C18 digested fraction exhibited greater antibacterial 569 activity against S. aureus bacteria with a minimum inhibition concentration (MIC) value of 1.0 570 \pm 0.1 mg/mL than the GCB fraction (MIC value of 3.5 \pm 0.1 mg/mL) (Table 5). Although a 571 total of 403 peptides from medium-sized peptide fraction and 572 peptides from the short-sized 572 peptide fraction were identified, none of these peptides pre-existed on BIOPEP or PeptideDB 573 databases. Interestingly, medium-size peptides were mostly hydrophilic with intermediate 574 polarity and small-sized peptide fractions were composed of mainly hydrophobic, less anionic 575 peptides, however, the combination of several anti-microbial peptides in the fraction may 576 induce a synergistic effect, inhibiting S. aureus activity more effectively than a single peptide. 577 Fraction 12 (MW not reported) purified from a bromelain hydrolysate of leatherjacket 578 (Meuchenia sp.) muscle protein by size using a RP-HPLC C-18 preparative column 579 demonstrated antimicrobial activities against gram-positive bacteria Bacillus cereus and S. 580 aureus with a MIC of 4.3 mg/mL (Table 5) (Salampessy et al., 2010). A study by Da Rocha et 581 al. (2018) included the anti-bacterial effect of Argentine croaker muscle protein hydrolysates on both gram-positive and gram-negative bacteria. Argentine croaker muscle protein 582 583 hydrolysates produced with either Alcalase® or Protamex® with DH 10% or 20%, all inhibited 584 gram-positive bacteria B. thermosphacta, L. innocua, and S. aureus. However, only Alcalase® 585 hydrolysates inhibited gram positive bacteria Listeria monocytogenes, and gram-negative 586 bacteria Yersinia enterecolitica. Alcalase® treated hydrolysates had a lower MW distribution 587 (<1285 Da), a higher content of hydrophobic AAs and as a result, more pronounced inhibition zones than Protamex® treated hydrolysates. Alcalase® hydrolysates dose-dependently 588 589 increased inhibition zones in Aeromonas hydrophila, B. thermospacta, Debaryomysces 590 hanseii, and L. innocua (1.25-7.5 mg/mL), with Alcalase® hydrolysed Argentine croaker 591 protein isolate at 10% DH inducing the greatest inhibition of D. hanseii (2.00-2.75 cm) and L.

592 innocua (1.25-1.50 cm) at 7.50 mg/mL (Table 5). According to Najafian and Babji (2012), 593 antimicrobial peptides are usually chains of less than 50 AAs in length of which nearly half are 594 hydrophobic with MW less than 10 kDa. Low MW fractions from tuna by-products were also 595 found to exhibit superior antimicrobial compared with larger fractions from the same source 596 (Gomez-Guillén et al., 2010, Pezeshk et al., 2019). Jemil et al. (2014) reported enhanced 597 resistance of gram-negative bacteria compared with gram-positive bacteria upon exposure to 598 FPHs. Fermented protein hydrolysates from sardinelle (Sardinella aurita) (SPH), zebra blenny 599 (Salaria basilisca) (ZPH), goby (Zosterizessor ophiocephalus) (GPH), and ray (Dasyatis 600 pastinaca) (RPH) and their antimicrobial activity against four gram-positive bacteria (S. 601 aureus, Microcossus luteus, B. cereus and Enterococcus faecalis) and five gram-negative 602 bacteria (E. coli, P aeruginosa, Klebsiella pneumonia, Salmonella enterica and Salmonella 603 typhi) was evaluated at 200 mg/mL (Table 5). SPH induced the greatest antibacterial effect of 604 the 4 hydrolysates with inhibition zones in all gram-positive bacteria and E. coli ranging from 605 10-24 mm. E. coli was also inhibited by ZPH, GPH and RPH; however, none of the 606 hydrolysates were successful in inhibiting any of the other four gram-negative bacteria. In 607 general, gram-negative bacteria have enhanced resistance to antimicrobial components due to 608 the presence of an outer membrane.

609 Antimicrobial FPHs/peptides present potential alternatives to conventional antibiotics 610 due to their broad-spectrum of activity and development of little to no pathogenic resistance 611 (Wang et al., 2016). They may also be suitable as bio-preservatives in food systems with the 612 aim of enhancing shelf life. The peptide Lys-Val-Glu-Ile-Val-Ala-Ile-Asn-Asp-Pro-Phe-Ile-613 Asp-Leu identified from Protamex® Atlantic mackerel hydrolysates was subsequently 614 synthesised and demonstrated anti-bacterial activity against food spoilage organisms Listeria 615 ivanovii and L. monocytogenes (MIC of 0.131 mM for both) (Offret et al., 2019). Although the 616 peptide also had an inhibitory effect on common human organisms M. luteus, Listeria

617 *acidophilus*, and *Bacteroides thetaiotaomicron*, MICs were half that of *Listeria* strains;
618 therefore, at equal concentrations, the peptide can prevent *Listeria* growth without impacting
619 normal human flora.

620

621 3.5 Anti-cancer

622 A limited number of studies have been published on the potential anti-cancer activity of 623 hydrolysates derived from fish muscle protein by assaying for anti-proliferative effect on 624 immortal cell lines. European seabass hydrolysate (1 mg/mL) prepared with chymotrypsin protease reduced cell viability in the human colon adenocarcinoma cell line, HT-29, by $39.6 \pm$ 625 626 12.8% (Altinelataman et al., 2019). Similar antiproliferative activity was observed for blue whiting protein hydrolysate (BWPH) (1mg/ mL) produced via hydrolysis with either 627 628 Protamex® or Alcalase® which induced a maximum 30% reduction and 27% reduction in 629 proliferation of breast cancer cells MDA-MB-231 and MCF-7/6 cells, respectively, after 72h 630 (Picot et al., 2006). Size exclusion chromatography confirmed the large MW distribution (100 631 Da-7 kDA) of BWPH indicating the presence of both free AAs and peptides which may have 632 been responsible for the superior anti-proliferative effect of BWPH over hydrolysates prepared 633 from salmon, emperor, pollack or siki. Similarly, solitary tunicate protein hydrolysates (1 634 mg/mL) inhibited growth of 3 human cancer cell lines; AGS (stomach cancer), DLD-1 (colon 635 cancer), and HeLa (cervical cancer). Solitary tunicate protein hydrolysate prepared with 636 Alcalase® demonstrated superior anti-proliferative activity than hydrolysates prepared with 637 thermoase or pepsin exhibiting IC₅₀ values of 1731.4 and 2922.5 μ g/mL for AGS cells and 638 HeLa cells, respectively. The Alcalase® hydrolysate was subsequently fractionated with the 639 resulting low MW fraction (fraction F₂: 3.6 kDa, rich in hydrophobic AAs (78.1%)) inhibiting cell growth of AGS, DLD-1 and HeLa cells with IC₅₀ values of 577.1, 1163.3, and 887.2 μ g/ 640 mL, respectively (Jumeri & Kim, 2011). It is possible that low MW peptides have enhanced 641

642 interactions with cancer cell components via enhanced cell mobility and diffusivity than large 643 MW components, thereby improving anti-cancer activity. Song and colleagues (2011) also 644 reported that thermal treatment (121°C, 30 min) of pepsin hydrolysate derived from half-fin 645 anchovy increased free AAs Val, Leu, Phe, His and Arg, increased the number of peptides with 646 MW distribution of 3000-5000 Da and <300 Da (p<0.005), and ultimately, increased antiproliferative activity against DU-145 human prostate cancer cells, 1299 human lung cancer 647 648 cells, and 109 human oesophagus cancer cells (p<0.05) compared to the non-heat sterilised 649 hydrolysate.

650 Hydrophobic AA residues are essential for the formation of a hydrophobic tail in the 651 COOH-terminal region, an attribute important for anti-cancer peptides. A recent review 652 discusses the classifications and structure-activity relationship of anti-cancer peptides in more 653 detail (Chiangjong et al., 2020). In short, in contrast to healthy cells, cancer cells possess 654 phosphatidylserine, a negatively charged phospholipid, allowing for electrostatic attraction 655 between cationic peptides and cancer cells. Upon electrostatic interaction with the membrane 656 surface of cancer cells, peptides arrange in either an α -helix or β -sheet, resulting in cell 657 membrane disruption. Anti-cancer peptides can be classified as i) molecularly targeted 658 peptides, ii) binding peptides, or iii) cell-stimulating peptides. Hydrophobic, positively charged 659 Lys- and Arg-rich peptides can induce cancer cell toxicity via disruption and penetration of 660 anionic, hydrophobic cancer cell membranes; a mechanism known as 'snorkeling'. The peptide 661 Tyr-Ala-Leu-Pro-Ala-His was subsequently purified from the heat-treated pepsin hydrolysate 662 of half-fin anchovy. Although this peptide inhibited prostate cancer PC-3 cells by 50% at 11.4 663 mg/mL, modification of the peptide to Tyr-Ala-Leu-Arg-Ala-His improved its inhibitory 664 activity (IC₅₀ value of 8.1 mg/mL). The enhanced cell permeation efficacy of Arg-rich peptides 665 may be due to the hydrogen-bond formation of guanidine moiety in Arg with phosphates, 666 sulfates, and carboxylates on cellular components (Song et al., 2014). Peptides Leu-Pro-HisVal-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr and Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr
purified from tuna dark muscle byproduct hydrolysates prepared with papain and Protease
XXIII induced a dose-dependent anti-proliferative effect on MCF-7 cells with IC₅₀ values of
8.1 and 8.8 μM, respectively (Hsu et al., 2011).

671 Further work on the isolation, identification, and elucidation of mechanism of action of 672 fish derived anti-cancer peptides is required. Furthermore, studies to-date used various cancer 673 cell lines to demonstrate the chemoprotective abilities of fish muscle protein hydrolysates and 674 peptides *in vitro*, however the majority of these studies lacked the inclusion of a non- cancerous 675 cell line controls. Nemipterus japonicus and Exocoetus volitans muscle hydrolysate fraction rich in Glu, Lys, Gly, and Thr induced a dose-dependent cytotoxic effect in the human 676 677 hepatoblastoma cell line, HepG2, with IC₅₀ values of 48.5 µg/mL and 21.6 µg/mL (Naqash & 678 Nazeer, 2010). Interestingly, neither fraction induced a cytotoxic effect in Vero (kidney 679 epithelial non-cancerous) cells.

680 If a peptide derived from fish is intended for use as a treatment for cancer, then similar to 681 all other peptide drugs it may face numerous limitations including peptide instability, poor 682 membrane permeability and poor oral bioavailability. Potential solutions to tackle these challenges include conjugation of therapeutic peptides with cell-penetrating peptides to 683 684 enhance transport across cellular membrane or conjugation with lipids, promoting 685 amphiphilicity, in turn, enhancing bioavailability, selectivity, potency, and membrane 686 penetration of peptide (Marqus et al., 2017). Peptide engineering via AA substitution or combination of peptides with each or other anti-cancer drugs may also promote improve 687 efficacy and efficiency of chemotherapy. 688

689 **3.6** Anti-obesity potential

690 Although enlargement of adipocytes is the main mechanism of weight gain in adults, obese 691 people generally have a higher amount of adipocytes than non-obese people. However, 692 adipocyte number is altered during childhood and adolescence and remains constant during 693 adulthood in both obese and lean people (Spalding et al., 2008). Adipogenesis is regulated by 694 various transcription factors including peroxisome proliferator-activated receptor γ (PPAR γ), 695 sterol regulatory element binding protein (SREBP)-1 and CCATT/enhancer binding protein a 696 (C/EBPa) (White & Stephans, 2010). After a thorough search of the relevant literature, no 697 adipocyte-modulating hydrolysate from a fish source was found. However, a peptide derived 698 from desalinated boiled tuna extract (Asp-Ile-Val-Asp-Lys-Ile-Glu-Ile) (5 mg/mL) reduced 699 triglyceride accumulation significantly (p<0.05) in differentiated adipocytes (3T3-L1 cell line) 700 compared with differentiated cells treated with media alone (Kim, Choi, Lee, & Nam, 2015). 701 This peptide also reduced expression levels of C-EBP α and PPAR γ , and expression levels of 702 adipogenic and lipogenic genes in differentiated 3T3-L1 cells. Pentapeptides Val-Ile-Asp-Pro-703 Trp and Ile-Arg-Trp-Trp (100 µM) purified from papain hydrolysed miiuy croaker muscle 704 (pH 7.5, 50°C, enzyme dose of 1.5%, 5h) significantly reduced oleic acid-induced lipid 705 accumulation in human liver carcinoma cells (HepG2) after 24h exposure (p<0.05 and p<0.01, 706 respectively) compared to the oleic acid model control (Wang et al., 2020). These 707 pentapeptides (100 µM) also reduced intracellular triglyceride levels (p<0.01 and p<0.001, 708 respectively), total cholesterol levels (p<0.01 and p<0.001, respectively), expression of 709 lipogenesis genes (SREBP-1c, SREBP-2, fatty acid synthase (FAS), acetyl-CoA carboxylase 710 (ACC), and 3-hydroxy-3-methyl-glutaryl-coenzyme-A reductase (HMGR)) and increased the 711 expression levels of lipolysis genes (PPARa, acyl-CoA oxidase 1 (ACOX-1), and carnitine 712 palmitoyltransferase-1 (CPT-1)) compared with cellular oleic acid model control. A database 713 search using BIOPEP-UWM revealed no sequence homology with known anti-adipogenic 714 peptides (http://www.uwm.edu.pl/biochemia/index.php/en/biopep). In addition, a peptide 715 structure-activity study by Pak et al. (2005) reported that the presence of Pro residues, Glu, 716 Thr, and Tyr side groups and hydrophobic regions promotes hypocholesterolemia via stabilisation of a 'turn' conformation and formation of hydrogen bonds to the binding site of 717 HMGR, a known rate limiting enzyme in cholesterol biosynthesis. Moreover, peptide 718 719 hydrophobicity is also correlated to their ability to bind to bile acids which may inhibit the 720 absorption of bile acids in the ileum and ultimately, decrease serum cholesterol levels (Pak et 721 al., 2005).

722 Alkaline protease hydrolysate (pH 11, 39°C, enzyme dosage 122 U/ mL) from water-723 soluble protein of crucian carp muscle increased inhibition of porcine pancreas lipase activity 724 *in vitro* as hydrolysis time increased, reaching a maximal value of $53.21 \pm 1.07\%$ at 10h (Liu 725 et al., 2013). Pancreatic lipase is the enzyme responsible for hydrolysis of 50-70% of total 726 dietary fat into monoglycerides, free fatty acids and other small molecules that are easily 727 absorbed by the intestine. Therefore, inhibition of pancreatic lipase activity can potentially 728 control energy intake. The alkaline protease hydrolysate from crucian carp also reduced α -729 amylase inhibitory activity by $20.07 \pm 0.87\%$.

730 In the quest to manage weight gain and reduce the incidence of obesity, food-derived 731 compounds have been identified that influence food intake pathways. Satiety hormones such 732 as Cholecystokinin (CCK), Glucagon-like peptide-1 (GLP-1) and Peptide YY (PYY) which 733 are released from enteroendocrine cells in response to food digestion have been shown to 734 suppress appetite and reduce food intake via activation of various signalization pathways. 735 Murine STC-1, murine GLUTag and human NCI-H716 cell lines are commonly used as 736 enteroendocrine models to screen for food components capable of inducing secretion of satiety 737 hormones.

738 Incubation of STC-1 cells for 2h with BWPH (hydrolysis conditions not reported) at 739 0.2% (w/v) and 1.0% (w/v) increased levels of CCK to 49.5 and 122.0 pM, respectively, 740 compared with STC-1 basal CCK levels (4.0 pM) (10 mM glucose) (Cudennec et al., 2008). 741 Although CCK-stimulating peptides were partially purified and characterised via size 742 exclusion chromatography to have an apparent MW ranging from 1000 to 1500 Da, the study 743 did not identify peptide sequences which may have influenced bioactivity. A follow-up study 744 determined BWPH (1.0% (w/v) for 2h incubation) also induced a 25-fold increase in GLP-1 745 concentration over basal (10 mM glucose) (Cudennec et al., 2012). In a study investigating the 746 antidiabetic activity of BWPH, Harnedy et al. (2018) also reported the ability of a BWPH 747 (prepared with Alcalase 2.4L and Flavourzyme 500L, pH 7.0, 50 °C, E/S ratio 0.74% (w/w)) 748 and a SGID digest to increase levels of GLP-1 significantly in GLUTag cells compared to the 749 glucose control (2 mM) (p<0.01 and p<0.001, respectively). An observation worth noting, 750 however, is that studies examining the satiety inducing effect of FPHs in vitro failed to include 751 assessment of the possible signalling mechanisms involved. Whereas, many studies 752 investigating milk and meat protein hydrolysates reported whether satiety hormone secretion 753 was induced via stimulation of the cyclic adenosine 3',5'-monophosphate (cAMP) pathway or 754 calcium signalling (Kondrashina et al., 2018; O'Halloran et al., 2018; Reimer, 2006). It should 755 also be noted that, to date, no study to the best of our knowledge, has identified and 756 characterised the peptide responsible for the satiating effect of FPHs. This information is 757 prerequisite for elucidating structure-function relationships and determining exact mechanisms 758 of action. In contrast, a number of GLP-1 and/ or CCK stimulating peptides have been 759 identified from milk and meat sources (Domenger et al., 2017; Komatsu et al., 2019; Tulipano 760 et al., 2011).

761 Albeit the majority of *in vitro* studies published to-date investigating the anti-obesity 762 activity of protein hydrolysates have focused on milk protein, it is difficult to compare the anti763 obesity potential of FPHs to other protein sources due to the methodological variation between 764 studies i.e., cell type, cell density, exposure time, hydrolysate concentration. However, a recent 765 review by Sharkey et al. (2020) concluded that many FPHs have potential to reduce body 766 weight and improve body composition in vivo and in clinical studies. The Norwegian Tromsø 767 Study is an epidemiological study with the focus of investigating the relationship between fish 768 consumption and the subsequent beneficial effects on metabolic syndrome. Data collected after a 13-year follow-up period (1994-1995, n = 23,907 to 2007–2008, n = 12,981) revealed that 769 770 consumption of lean fish once a week or more was associated with decreased future metabolic 771 score, decreased triglyceride content, and increased high-density lipoprotein-cholesterol, 772 whereas decreased waist circumference and blood pressure was identified only for men (Tørris 773 et al., 2017).

774

775 4 Applications, challenges and future perspectives

776 FPHs represent desirable functional food ingredients owing to their beneficial impact on both 777 health and food quality. Addition of antioxidant or anti-microbial FPHs to a food system may 778 inhibit lipid peroxidation or growth of food spoilage microorganisms, respectively, thereby 779 potentially extending shelf life of supplemented products. Not only do FPH have use as 780 preservative ingredients, but some FPH are also commercialised functional food ingredients 781 with health promoting claims. BWPH which induced CCK and GLP-1 secretion in STC-1 cells, 782 was subsequently demonstrated to increase plasma concentrations of CCK and GLP-1, improve 783 body composition and reduce body weight upon oral administration (1.4 g) to 120 overweight $(25 \text{ kg/m}^2 \le \text{body mass index (BMI)} < 30 \text{ kg/m}^2)$ adults over 90 days. BWPH is now 784 commercialised and marketed as Slimpro[®] (Nobile et al., 2016). In addition, peptides purified 785 786 from dried bonito (katsuobushi) via thermolysin digestion exhibiting ACE-inhibitory activities

in vitro were also shown to exhibit anti-hypertensive effects in spontaneously hypertensive rats
and borderline (high normal) and mildly hypertensive adults (1.4 g/ day orally administrated
over 5 weeks) (Fujita et al., 2001; Yokoyama et al., 1992). *Katsuobushi* oligopeptide received
official approval as Foods for Specific Health Use (FoSHU) in 1999 by the Ministry of Health
and Welfare in Japan for prevention of hypertension in at-risk individuals.

792 Although this review discusses only six potential bioactivities of fish muscle protein 793 hydrolysates in vitro, numerous novel bioactivities have emerged in recent years. FPHs have 794 recently been shown to enhance cognitive memory, promote skin repair and regeneration, and 795 increase post-exercise aminoacidemia, as well as the ability to increase bone mass with 796 potential to treat osteoporosis and bone loss (Cordeiro et al., 2020; Lee et al., 2019; Zhang, 797 Zhang, et al., 2018; Zhang, Su, et al., 2019). There are now vast possible applications for 798 bioactive FPHs which are garnering more and more interest from food, pharmaceutical and 799 cosmetic industries.

Although bioactive fish hydrolysates and peptides are generally not as potent as synthetic drugs, they could provide a safe and natural alternative for the prevention more than the treatment of disease. However, for bioactive efficacy in a functional food offering, fish hydrolysates similar to other food hydrolysates must overcome several hurdles not least of which includes issues of processing, food formulation, sensory acceptance, survival during GI digestion and bioavailability.

A systematic approach for optimization of the numerous parameters which influence the production of bioactive peptides is now advised compared to 'one factor at a time' or 'trial and error' approaches which should now be deemed obsolete (Chakrabarti et al., 2018). The development of bioinformatics analysis in recent years has promoted a highly useful approach for the generation of bioactive peptides via the utilization of computational data to predict peptide sequences likely to induce specific bioactivities and elucidate structure-function relationships. Peptide databases should be exploited to save time and expenses involved in purifying fish peptides and testing various potential bioactivities. These databases also provide information about peptide structure-function relationships, molecular docking, and peptidereceptor interactions, which are essential for the development of therapeutic products.

Although *in vitro* investigations offer great insight into the potential bioactivities of FPH, for FPHs to have use as bioactive agents with the aim to improve human health, more clinical trials are required in order to determine FPH bioavailability and absorption through the GI barrier ensuring eventual contact with target cells. In addition, future studies should elucidate mechanisms of action of bioactive fish muscle protein hydrolysates, as well as, identify individual bioactive peptides from fish protein fractions so that peptide structurefunction relationships can be further understood.

823

824 5 Conclusion

825

826 This review has discussed a plethora of biologically active protein hydrolysates (and 82 827 bioactive peptides) prepared from muscle of various fish species and highlighted the 828 relationship between peptide structure and exhibited in vitro bioactivity. Croaker fish 829 (Sciaenidae family) muscle appears to be the most common substrate for generation of 830 antioxidant, anti-inflammatory, and anti-microbial protein hydrolysates and peptides; whereas, 831 the majority of ACE-inhibitory hydrolysates discussed were prepared from salmon muscle. 832 Microbial proteases including Alcalase®, Protamex® and Flavourzyme® are generally the 833 enzymes of choice to produce potent fish muscle protein hydrolysates. Alcalase® hydrolysed 834 Argentine croaker myofibrillar protein hydrolysate represented the most multifunctional fish 835 muscle hydrolysate demonstrating antioxidant, anti-inflammatory, and anti-microbial activities 836 (Da Rocha et al., 2018). Due to the range of biofunctionalities exhibited, the hydrolysate 837 possesses enhanced potential as a value-added ingredient for application in functional foods 838 and nutraceutical products. However, the peptide responsible for exhibited bioactivities must 839 be identified prior to commercialisation. It is possible that free AAs released during hydrolysis 840 may also influence bioactivity. Short-chain peptides commonly exhibit bioactivity in vivo as 841 they are too small to act as a substrate for digestive proteases, thereby have heightened 842 resistance to GI digestion and increased probability of crossing the intestinal barrier to elicit 843 their biological function. However, similar to other food-derived protein hydrolysates, fish 844 hydrolysates have also failed efficacy assessments in follow-up in vivo trials (Chai et al., 2016; 845 Giannetto et al., 2020)

This review offers a comparison of studies investigating the cellular *in vitro* bioactivity of fish muscle protein hydrolysates and has collated numerous data informing the reader of suitable protein sources, enzymes, and processing conditions for the generation of bioactive hydrolysates. This review thereby may be a useful data base when designing future studies on fish muscle protein hydrolysates/peptides *in vitro* or *in vivo* as no database listing exclusively bioactive peptides derived from fish sources currently exists.

Overall, ACE inhibition appears to be the most promising bioactivity of fish muscle protein hydrolysates/ peptides of the six bioactivities discussed in this review. Although hydrolysates from other protein sources such as milk, meat and plant show similar inhibitory activity, fish is now an abundant source of protein due to the 'landing obligation' policy which was introduced by the European Commission with the aim of progressive elimination of discards in all EU fisheries. Fish protein now represents an affordable alternative to milk proteins which are currently the main source of bioactive peptides.

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864

865 **Declaration of Competing Interest**

866 The authors declare no conflict of interest

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868 **References**:

869

870Altınelataman, C., Koroleva, O., Fedorova, T., Torkova, A., Lisitskaya, K., Tsentalovich, M., . . .

871Çelik, U. (2019). An in vitro and in silico study on the antioxidant and cell culture-based

study on the chemoprotective activities of fish muscle protein hydrolysates obtained from

873 European seabass and gilthead seabream. *Food Chem*, 271, 724-732.
874 <u>https://doi.org/10.1016/j.foodchem.2018.08.004</u>.

875Auwal, S. M., Zarei, M., Abdul-Hamid, A., & Saari, N. (2017). Response Surface Optimisation

876 for the Production of Antioxidant Hydrolysates from Stone Fish Protein Using Bromelain.

877 Evid Based Complement Alternat Med, 2017, 4765463.
 878 <u>https://doi.org/10.1155/2017/4765463.</u>

879Balti, R., Bougatef, A., Sila, A., Guillochon, D., Dhulster, P., & Nedjar-Arroume, N. (2015). Nine 880 novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (Sepia officinalis) muscle protein hydrolysates and antihypertensive effect of the potent active 881 882 peptide in spontaneously hypertensive rats. Food Chem, 170, 519-525. 883 https://doi.org/10.1016/j.foodchem.2013.03.091.

884Bashir, K. M. I., Park, Y.-J., An, J. H., Choi, S.-J., Kim, J.-H., Baek, M.-K., ... Choi, J.-S. (2018).

885 Antioxidant Properties of Scomber japonicus Hydrolysates Prepared by Enzymatic

886 Hydrolysis. Journal of Aquatic Food Product Technology, 27(1), 107-121.

887 <u>https://doi.org/10.1080/10498850.2017.1407013</u>.

888Bhandari, D., Rafiq, S., Gat, Y., Gat, P., Waghmare, R., & Kumar, V. (2020). A Review on
Bioactive Peptides: Physiological Functions, Bioavailability and Safety. *International Journal of Peptide Research and Therapeutics,* 26(1), 139-150.
<u>https://doi.org/10.1007/s10989-019-09823-5</u>.

892Bkhairia, I., Ben Slama Ben Salem, R., Nasri, R., Jridi, M., Ghorbel, S., & Nasri, M. (2016). Invitro antioxidant and functional properties of protein hydrolysates from golden grey mullet
prepared by commercial, microbial and visceral proteases. *Journal of food science and technology, 53*(7), 2902-2912. <u>https://doi.org/10.1007/s13197-016-2200-5</u>.

896Cerrato, A., Capriotti, A.L., Capuano, F., Cavaliere, C., Montone, A.M.I., Montone, C.M.,
Piovesana, S., Zenezini Chiozzi, R. and Laganà, A. (2020). Identification and
Antimicrobial Activity of Medium-Sized and Short-Peptides from Yellowfin Tuna
(Thunnus albacares) Simulated Gastrointestinal Digestion. *Foods*, 9(9), p.1185.
https://doi.org/10.3390/foods9091185

901Chai, H.-J., Chan, Y.-L., Li, T.-L., Shiau, C.-Y., & Wu, C.-J. (2013). Evaluation of lanternfish

902 (Benthosema pterotum) hydrolysates as antioxidants against hydrogen peroxide induced
903 oxidative injury. *Food Research International*, 54(2), 1409-1418.
904 <u>https://doi.org/https://doi.org/10.1016/j.foodres.2013.09.052</u>.

905Chai, H.-J., Wu, C.-J., Yang, S.-H., Li, T.-L., & Sun Pan, B. (2016). Peptides from hydrolysate of

lantern fish (Benthosema pterotum) proved neuroprotective in vitro and in vivo. *Journal of*

907 *Functional Foods, 24*, 438-449. <u>https://doi.org/https://doi.org/10.1016/j.jff.2016.04.009</u>.

908Chakrabarti, S., Guha, S., & Majumder, K. (2018). Food-Derived Bioactive Peptides in Human

909 Health: Challenges and Opportunities. Nutrients, 10(11).
910 https://doi.org/10.3390/nu10111738.

911Chi, C.-F., Wang, B., Deng, Y.-Y., Wang, Y.-M., Deng, S.-G., & Ma, J.-Y. (2014). Isolation and
characterization of three antioxidant pentapeptides from protein hydrolysate of monkfish

913 (Lophius litulon) muscle. Food Research International, 55, 222-228.

914 <u>https://doi.org/https://doi.org/10.1016/j.foodres.2013.11.018</u>.

915Chiangjong, W., Chutipongtanate, S. and Hongeng, S. (2020). Anticancer peptide:
916 Physicochemical property, functional aspect and trend in clinical application. *International Journal of Oncology*, *57*(3), 678-696. https://doi.org/10.3892/ijo.2020.5099

918Chel-Guerrero, L., Estrella-Millán, Y., Betancur-Ancona, D., Aranda-González, I., CastellanosRuelas, A. and Gallegos-Tintoré, S. (2020). Antioxidant, chelating, and angiotensinconverting enzyme inhibitory activities of peptide fractions from red lionfish (Pterois

921 volitans L.) muscle protein hydrolysates. *International Food Research Journal*, 27(2).

922Chen, J., Wang, Y., Zhong, Q., Wu, Y., & Xia, W. (2012). Purification and characterization of a

923 novel angiotensin-I converting enzyme (ACE) inhibitory peptide derived from enzymatic

924 hydrolysate of grass carp protein. *Peptides*, 33(1), 52-58.
925 https://doi.org/https://doi.org/10.1016/j.peptides.2011.11.006.

926Cordeiro, E. M., de Oliveira, G. V., Volino-Souza, M., Velozo, O. d. C., & Alvares, T. S. (2020).

927 Effects of fish protein hydrolysate ingestion on postexercise aminoacidemia compared with

928 whey protein hydrolysate in young individuals. *Journal of Food Science*, 85(1), 21-27.

929 https://doi.org/https://doi.org/10.1111/1750-3841.14970.

930Cudennec, B., Fouchereau-Peron, M., Ferry, F., Duclos, E., & Ravallec, R. (2012). In vitro and in

931 vivo evidence for a satiating effect of fish protein hydrolysate obtained from blue whiting

932 (Micromesistius poutassou) muscle. Journal of Functional Foods, 4(1), 271-277.

933 https://doi.org/https://doi.org/10.1016/j.jff.2011.12.003.

934Cudennec, B., Ravallec-Plé, R., Courois, E., & Fouchereau-Peron, M. (2008). Peptides from fish
935 and crustacean by-products hydrolysates stimulate cholecystokinin release in STC-1 cells.

936 Food Chemistry, 111(4), 970-975.

937 https://doi.org/https://doi.org/10.1016/j.foodchem.2008.05.016.

938Cumby, N., Zhong, Y., Naczk, M. and Shahidi, F., 2008. Antioxidant activity and water-holding
939 capacity of canola protein hydrolysates. *Food chemistry*, *109*(1), pp.144-148.
940 https://doi.org/10.1016/j.foodchem.2007.12.039

941Da Rocha, M., Alemán, A., Baccan, G. C., López-Caballero, M. E., Gómez-Guillén, C., Montero,

- 942 P., & Prentice, C. (2018). Anti-Inflammatory, Antioxidant, and Antimicrobial Effects of
- 943 Underutilized Fish Protein Hydrolysate. Journal of Aquatic Food Product Technology,

944 27(5), 592-608. https://doi.org/10.1080/10498850.2018.1461160.

945Dallas, D. C., Sanctuary, M. R., Qu, Y., Khajavi, S. H., Van Zandt, A. E., Dyandra, M., . . . German,

J. B. (2017). Personalizing protein nourishment. Crit Rev Food Sci Nutr, 57(15), 3313-

947 3331. https://doi.org/10.1080/10408398.2015.1117412.

948Darewicz, M., Borawska, J., Vegarud, G. E., Minkiewicz, P., & Iwaniak, A. (2014). Angiotensin
949 I-converting enzyme (ACE) inhibitory activity and ACE inhibitory peptides of salmon
950 (Salmo salar) protein hydrolysates obtained by human and porcine gastrointestinal
951 enzymes. *International journal of molecular sciences, 15*(8), 14077-14101.
952 <u>https://doi.org/10.3390/ijms150814077</u>.

953Dauksas, E., Slizyte, R., Rustad, T. and Storro, I. (2004). Bitterness in fish protein hydrolysates

and methods for removal. Journal of Aquatic Food Product Technology, 13(2), pp.101-

955 114. <u>https://doi.org/10.1300/J030v13n02_09</u>

956Domenger, D., Caron, J., Belguesmia, Y., Lesage, J., Dhulster, P., Ravallec, R., & Cudennec, B.
(2017). Bioactivities of hemorphins released from bovine haemoglobin gastrointestinal
digestion: Dual effects on intestinal hormones and DPP-IV regulations. *Journal of Functional Foods, 36*, 9-17. <u>https://doi.org/https://doi.org/10.1016/j.jff.2017.06.047</u>.
960Elavarasan, K., & Shamasundar, B. A. (2017). Antioxidant and emulsion properties of freshwater

961 carps (Catla catla, Labeo rohita, Cirrhinus mrigala) protein hydrolysates prepared using

962 flavorzyme. Food Science and Biotechnology, 26(5), 1169-1176.
963 https://doi.org/10.1007/s10068-017-0154-7.

964Elavarasan, K., Shamasundar, B. A., Badii, F., & Howell, N. (2016). Angiotensin I-converting
965 enzyme (ACE) inhibitory activity and structural properties of oven- and freeze-dried
966 protein hydrolysate from fresh water fish (Cirrhinus mrigala). *Food Chemistry, 206*, 210967 216. <u>https://doi.org/https://doi.org/10.1016/j.foodchem.2016.03.047</u>.

968Enari, H., Takahashi, Y., Kawarasaki, M., Tada, M., & Tatsuta, K. (2008). Identification of
angiotensin I-converting enzyme inhibitory peptides derived from salmon muscle and their
antihypertensive effect. *Fisheries Science*, *74*(4), 911-920. <u>https://doi.org/10.1111/j.1444-</u>
2906.2008.01606.x.

972Fonseca, R. A. S., Silva, C. B. M., Fernandes, G., & Prentice, C. (2016). Enzymatic hydrolysis of
cobia (Rachycentron canadum) meat and wastes using different microbial enzymes. *International Food Research Journal, 23*, 152-160.

975Fujita, H., Yamagami, T., & Ohshima, K. (2001). Effects of an ace-inhibitory agent, katsuobushi
oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly
hypertensive subjects1 1Abbreviations: KO: katsuobushi oligopeptide; ACE: angiotensin
I-converting enzyme; SHR: spontaneously hypertensive rat; IC50: 50% inhibitory
concentration; LKPNM: Leu-Lys-Pro-Asn-Met; SBP: systolic blood pressure; DBP:
diastolic blood pressure; HHL: hippuryl-histidyl-leucine. *Nutrition Research, 21*(8), 1149-

981 1158. <u>https://doi.org/https://doi.org/10.1016/S0271-5317(01)00333-5</u>.

982Gao, R., Shu, W., Shen, Y., Sun, Q., Bai, F., Wang, J., Li, D., Li, Y., Jin, W. and Yuan, L. (2020).

983 Sturgeon protein-derived peptides exert anti-inflammatory effects in LPS-stimulated

984 RAW264. 7 macrophages via the MAPK pathway. Journal of Functional Foods, 72,

985 p.104044. <u>https://doi.org/10.1016/j.jff.2020.104044</u>

41

986Ghassem, M., Babji, A. S., Said, M., Mahmoodani, F., & Arihara, K. (2014). Angiotensin I–
087 Converting Enzyme Inhibitory Peptides from Snakehead Fish Sarcoplasmic Protein
088 Hydrolysate. *Journal of Food Biochemistry*, 38(2), 140-149.
089 <u>https://doi.org/https://doi.org/10.1111/jfbc.12031</u>.

990Giannetto, A., Esposito, E., Lanza, M., Oliva, S., Riolo, K., Di Pietro, S., . . . Macrì, F. (2020).

Protein Hydrolysates from Anchovy (Engraulis encrasicolus) Waste: In Vitro and In Vivo
Biological Activities. *Marine drugs*, 18(2), 86. <u>https://doi.org/10.3390/md18020086</u>.

993Godinho, I., Pires, C., Pedro, S., Teixeira, B., Mendes, R., Nunes, M. L., & Batista, I. (2016).
994 Antioxidant Properties of Fish Protein Hydrolysates Prepared from Cod Protein
995 Hydrolysate by Bacillus sp. *Appl Biochem Biotechnol, 178*(6), 1095-1112.
996 <u>https://doi.org/10.1007/s12010-015-1931-5.</u>

997Gomez-Guillén, G., López-Caballero, M.E., Alemán, A., López de Lacey, A., Giménez, B., &
Montero, P. (2010). Antioxidant and antimicrobial peptide fractions from squid and tuna
skin gelatin, in E. Le Bihan, (Ed.) *Sea By-Products as Real Material: New Ways of Application*. Kerala: Transworld Research Network. pp.89-115

1001Gómez-Ruiz, J.Á., Taborda, G., Amigo, L., Recio, I. and Ramos, M. (2006). Identification of ACE-

1002 inhibitory peptides in different Spanish cheeses by tandem mass spectrometry. *European*

1003 Food Research and Technology, 223(5), pp.595-601. https://doi.org/10.1007/s00217-005-

1004 <u>0238-0</u>

1005Ha, N. C., Hien, D. M., Thuy, N. T., Nguyen, L. T., & Devkota, L. (2017). Enzymatic Hydrolysis
of Catfish (Pangasius hypophthalmus) By-Product: Kinetic Analysis of Key Process
Parameters and Characteristics of the Hydrolysates Obtained. *Journal of Aquatic Food*

1008 Product Technology, 26(9), 1070-1082. <u>https://doi.org/10.1080/10498850.2017.1376027</u>.

1009Hamed, I., Özogul, F., Özogul, Y., & Regenstein, J. M. (2015). Marine Bioactive Compounds and
Their Health Benefits: A Review. *Comprehensive Reviews in Food Science and Food Safety*, 14(4), 446-465. https://doi.org/https://doi.org/10.1111/1541-4337.12136.

1012Harnedy, P. A., Parthsarathy, V., McLaughlin, C. M., O'Keeffe, M. B., Allsopp, P. J., McSorley,

1013 E. M., ... FitzGerald, R. J. (2018). Blue whiting (Micromesistius poutassou) muscle protein

1014 hydrolysate with in vitro and in vivo antidiabetic properties. *Journal of Functional Foods*,

1015 *40*, 137-145. <u>https://doi.org/https://doi.org/10.1016/j.jff.2017.10.045</u>.

1016Hayes, M. and Flower, D. (2013). Bioactive peptides from marine processing byproducts, in B.

1017 Hernández-Ledesma, & M. Herrero (Eds.), *Bioactive Compounds from Marine Foods:*

1018 Plant and Animal Sources (pp.57-71). Chicago: John Wiley & Sons.

1019He, Y., Pan, X., Chi, C.-F., Sun, K.-L., & Wang, B. (2019). Ten new pentapeptides from protein

1020 hydrolysate of miiuy croaker (Miichthys miiuy) muscle: Preparation, identification, and

1021 antioxidant activity evaluation. *LWT*, 105,

1022 <u>https://doi.org/https://doi.org/10.1016/j.lwt.2019.01.054</u>.

1023Hsu, K.-C., Li-Chan, E. C. Y., & Jao, C.-L. (2011). Antiproliferative activity of peptides prepared

1024 from enzymatic hydrolysates of tuna dark muscle on human breast cancer cell line MCF-

 1025
 7.
 Food
 Chemistry,
 126(2),
 617-622.

1026 <u>https://doi.org/https://doi.org/10.1016/j.foodchem.2010.11.066</u>.

1027Hu, X. M., Wang, Y. M., Zhao, Y. Q., Chi, C. F., & Wang, B. (2020). Antioxidant Peptides from

1028 the Protein Hydrolysate of Monkfish (Lophius litulon) Muscle: Purification, Identification,

- and Cytoprotective Function on HepG2 Cells Damage by H(2)O(2). *Marine drugs, 18*(3).
- 1030 <u>https://doi.org/10.3390/md18030153</u>.

1031Ishibashi, N., Kouge, K., Shinoda, I., Kanehisa, H., & Okai, H. (1988). A mechanism for bitter

taste sensibility in peptides. Agricultural and Biological Chemistry, 52(3), 819-827.

1033 <u>https://doi.org/10.1080/00021369.1988.10868743</u>

1-8.

1034Jain, A. K., Singh, D., Dubey, K., Maurya, R., Mittal, S., & Pandey, A. K. (2018). Chapter 3 -

- Models and Methods for In Vitro Toxicity. In A. Dhawan & S. Kwon (Eds.), *In Vitro Toxicology* (pp. 45-65): Academic Press.
- 1037Je, J. Y., & Kim, S. K. (2012). Chitosan as potential marine nutraceutical. Adv Food Nutr Res, 65,
- 1038 121-135. <u>https://doi.org/10.1016/b978-0-12-416003-3.00007-x</u>.
- 1039Jemil, I., Jridi, M., Nasri, R., Ktari, N., Ben Slama-Ben Salem, R., Mehiri, M., ... Nasri, M. (2014).
- 1040 Functional, antioxidant and antibacterial properties of protein hydrolysates prepared from
- 1041 fish meat fermented by Bacillus subtilis A26. *Process Biochemistry*, 49(6), 963-972.

1042 <u>https://doi.org/https://doi.org/10.1016/j.procbio.2014.03.004</u>.

- 1043 Jiang, H., Tong, T., Sun, J., Xu, Y., Zhao, Z., & Liao, D. (2014). Purification and characterization
- 1044of antioxidative peptides from round scad (Decapterus maruadsi) muscle protein1045hydrolysate.FoodChemistry,154,158-163.1046https://doi.org/https://doi.org/10.1016/j.foodchem.2013.12.074.
- 1047Jiang, Z., Zhang, H., Bian, X., Li, J., Li, J., & Zhang, H. (2019). Insight into the binding of ACE-
- inhibitory peptides to angiotensin-converting enzyme: A molecular simulation. *Molecular Simulation*, 45(3), 215-222
- 1050Jumeri, & Kim, S. M. (2011). Antioxidant and anticancer activities of enzymatic hydrolysates of
- 1051 solitary tunicate (Styela clava). Food Science and Biotechnology, 20(4), 1075.
- 1052 <u>https://doi.org/10.1007/s10068-011-0146-y</u>.

1053Kangsanant, S., Murkovic, M., & Thongraung, C. (2014). Antioxidant and nitric oxide inhibitory
activities of tilapia (Oreochromis niloticus) protein hydrolysate: effect of ultrasonic
pretreatment and ultrasonic-assisted enzymatic hydrolysis. *International Journal of Food Science* & *Technology*, 49(8), 1932-1938.
https://doi.org/https://doi.org/10.1111/ijfs.12551.

1058Kangsanant, S., Thongraung, C., Jansakul, C., Murkovic, M., & Seechamnanturakit, V. (2015).

1059 Purification and characterisation of antioxidant and nitric oxide inhibitory peptides from

1060 Tilapia (Oreochromis niloticus) protein hydrolysate. International Journal of Food Science

1061 & Technology, 50(3), 660-665. <u>https://doi.org/https://doi.org/10.1111/ijfs.12680</u>.

1062Kim, S., & Byun, H.-G. (2012). The Novel Angiotensin I Converting Enzyme Inhibitory Peptide

1063 from Rainbow Trout Muscle Hydrolysate. *Fisheries and Aquatic Sciences*, 15, 183-190.

1064 https://doi.org/10.5657/FAS.2012.0183

1065Kim, Y.-M., Kim, I.-H., Choi, J.-W., Lee, M.-K., & Nam, T.-J. (2015). The anti-obesity effects of

a tuna peptide on 3T3-L1 adipocytes are mediated by the inhibition of the expression of

1067 lipogenic and adipogenic genes and by the activation of the Wnt/ β -catenin signaling

1068 pathway. International journal of molecular medicine, 36(2), 327-334.

1069 <u>https://doi.org/10.3892/ijmm.2015.2231</u>.

1070Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and

1071 functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis)

as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, 102(4), 1317-

1073 1327. <u>https://doi.org/https://doi.org/10.1016/j.foodchem.2006.07.016</u>.

1074Ko, J. Y., Lee, J. H., Samarakoon, K., Kim, J. S., & Jeon, Y. J. (2013). Purification and

1075 determination of two novel antioxidant peptides from flounder fish (Paralichthys olivaceus)

 1076
 using digestive proteases.
 Food
 Chem
 Toxicol,
 52,
 113-120.

 1077
 https://doi.org/10.1016/j.fct.2012.10.058.

1078Ko, S. C., & Jeon, Y. J. (2015). Anti-inflammatory effect of enzymatic hydrolysates from Styela
clava flesh tissue in lipopolysaccharide-stimulated RAW 264.7 macrophages and in vivo
zebrafish model. *Nutr Res Pract*, 9(3), 219-226. https://doi.org/10.4162/nrp.2015.9.3.219.

1081Komatsu, Y., Wada, Y., Izumi, H., Shimizu, T., Takeda, Y., Hira, T., & Hara, H. (2019). Casein

1082 materials show different digestion patterns using an in vitro gastrointestinal model and

different release of glucagon-like peptide-1 by enteroendocrine GLUTag cells. *Food Chemistry*, 277, 423-431. https://doi.org/https://doi.org/10.1016/j.foodchem.2018.10.123.

1085Kondrashina, A., Papkovsky, D., & Giblin, L. (2018). Physiological Gut Oxygenation Alters GLP-

1086 1 Secretion from the Enteroendocrine Cell Line STC-1. *Molecular Nutrition & Food* 1087 *Research, 62*(3), 1700568. https://doi.org/https://doi.org/10.1002/mnfr.201700568.

1088Kumar, J., Teoh, S. L., Das, S., & Mahakknaukrauh, P. (2017). Oxidative Stress in Oral Diseases:

- Understanding Its Relation with Other Systemic Diseases. *Frontiers in physiology*, *8*, 693693. https://doi.org/10.3389/fphys.2017.00693.
- 1091Lee, H. J., Jang, H. L., Ahn, D. K., Kim, H. J., Jeon, H. Y., Seo, D. B., . . . Kang, S. S. (2019).

1092 Orally administered collagen peptide protects against UVB-induced skin aging through the

absorption of dipeptide forms, Gly-Pro and Pro-Hyp. *Biosci Biotechnol Biochem*, 83(6),

1094 1146-1156. <u>https://doi.org/10.1080/09168451.2019.1580559</u>.

1095Lee, S. Y., & Hur, S. J. (2017). Antihypertensive peptides from animal products, marine organisms,

- 1096
 and
 plants.
 Food
 Chemistry,
 228,
 506-517.

 1097
 https://doi.org/10.1016/j.foodchem.2017.02.039.
- 1098Leni, G., Soetemans, L., Caligiani, A., Sforza, S., & Bastiaens, L. (2020). Degree of hydrolysis
 affects the techno-functional properties of lesser mealworm protein
 hydrolysates. *Foods*, 9(4), 381.<u>https://doi.org/10.3390/foods9040381</u>
- 1101Li, X., Luo, Y., Shen, H., & You, J. (2012). Antioxidant activities and functional properties of
- 1102 grass carp (Ctenopharyngodon idellus) protein hydrolysates. J Sci Food Agric, 92(2), 292-
- 1103 298. <u>https://doi.org/10.1002/jsfa.4574</u>.
- 1104Lima, M.M., Vanier, N.L., Dias, A.R.G., Zavareze, E., Prentice, C., & Moreira, A.D.S. (2019).
- 1105 Whitemouth croaker (Micropogonias furnieri) protein hydrolysates: chemical composition,
- 1106 molecular mass distribution, antioxidant activity and amino acid profile. *International*
- 1107 *Food Research Journal*, *26*(1), pp.247-254.

1108Liu, J., Lyu, F., Zhou, X., Wang, B., Wang, X., & Ding, Y. (2015). Preparation of Skipjack Tuna
(Katsuwonus pelamis) Protein Hydrolysate Using Combined Controlled Enzymatic
Hydrolysis and Glycation for Improved Solubility and Emulsifying Properties. *Journal of Food and Nutrition Research*, 3(7), 471-477. https://doi.org/10.12691/jfnr-3-7-9.

1112Liu, L., Wang, Y., Peng, C., & Wang, J. (2013). Optimization of the preparation of fish protein

1113 anti-obesity hydrolysates using response surface methodology. International journal of

1114 *molecular sciences*, *14*(2), 3124-3139. <u>https://doi.org/10.3390/ijms14023124</u>.

1115Manzanares, P., Gandía, M., Garrigues, S., & Marcos, J. F. (2019). Improving Health-Promoting

1116 Effects of Food-Derived Bioactive Peptides through Rational Design and Oral Delivery

1117 Strategies. *Nutrients*, *11*(10). <u>https://doi.org/10.3390/nu11102545</u>.

1118Marqus, S., Pirogova, E. and Piva, T.J., 2017. Evaluation of the use of therapeutic peptides for
cancer treatment. *Journal of biomedical science*, 24(1), 1-15.
<u>https://doi.org/10.1186/s12929-017-0328-x</u>

1121Maruyama, S., Mitachi, H., Tanaka, H., Tomizuka, N., & Suzuki, H. (1987). Studies on the Active
Site and Antihypertensive Activity of Angiotensin I-Converting Enzyme Inhibitors
Derived from Casein. *Agricultural and Biological Chemistry*, 51(6), 1581-1586.

1124 <u>https://doi.org/10.1080/00021369.1987.10868244</u>.

1125Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial

1126 peptides: Their production, assessment, and applications. *Peptides*, 33(1), 178-185.

1127 <u>https://doi.org/10.1016/j.peptides.2011.11.013</u>.

1128Nakajima, K., Yoshie-Stark, Y., & Ogushi, M. (2009). Comparison of ACE inhibitory and DPPH

radical scavenging activities of fish muscle hydrolysates. Food Chemistry, 114(3), 844-

1130 851. <u>https://doi.org/https://doi.org/10.1016/j.foodchem.2008.10.083</u>.

1131Naqash, S. Y., & Nazeer, R. A. (2010). Antioxidant Activity of Hydrolysates and Peptide Fractions

1132 of Nemipterus japonicus and Exocoetus volitans Muscle. Journal of Aquatic Food Product

1133 *Technology*, *19*(3-4), 180-192. <u>https://doi.org/10.1080/10498850.2010.506256</u>.

1134Nasri, M. (2017). Chapter Four - Protein Hydrolysates and Biopeptides: Production, Biological

1135 Activities, and Applications in Foods and Health Benefits. A Review. In F. Toldrá (Ed.),

1136 *Advances in Food and Nutrition Research* (pp. 109-159): Academic Press.

1137Nasri, R., Jridi, M., Lassoued, I., Jemil, I., Ben Slama-Ben Salem, R., Nasri, M., & Karra-

1138 Châabouni, M. (2014). The influence of the extent of enzymatic hydrolysis on antioxidative

properties and ACE-inhibitory activities of protein hydrolysates from goby (Zosterisessor

- 1140 ophiocephalus) muscle. Appl Biochem Biotechnol, 173(5), 1121-1134.
- 1141 <u>https://doi.org/10.1007/s12010-014-0905-3</u>.

1142Nasri, R., Younes, I., Jridi, M., Trigui, M., Bougatef, A., Nedjar-Arroume, N., . . . KarraChâabouni, M. (2013). ACE inhibitory and antioxidative activities of Goby (Zosterissessor

1144 ophiocephalus) fish protein hydrolysates: Effect on meat lipid oxidation. *Food Research*

- 1145 International, 54(1), 552-561.
- 1146 <u>https://doi.org/https://doi.org/10.1016/j.foodres.2013.07.001</u>.

1147Nazeer, R. A., Kumar, N. S., & Ganesh, R. J. (2012). In vitro and in vivo studies on the antioxidant

activity of fish peptide isolated from the croaker (Otolithes ruber) muscle protein

1149 hydrolysate. *Peptides*, 35(2), 261-268. <u>https://doi.org/10.1016/j.peptides.2012.03.028</u>

1150Nesse, K. O., Nagalakshmi, A. P., Marimuthu, P., & Singh, M. (2011). Efficacy of a fish protein

1151 hydrolysate in malnourished children. *Indian journal of clinical biochemistry : IJCB, 26*(4),

- 1152 360-365. <u>https://doi.org/10.1007/s12291-011-0145-z</u>.
- 1153Nobile, V., Duclos, E., Michelotti, A., Bizzaro, G., Negro, M., & Soisson, F. (2016).
 Supplementation with a fish protein hydrolysate (Micromesistius poutassou): effects on

body weight, body composition, and CCK/GLP-1 secretion. *Food Nutr Res, 60*, 29857.
https://doi.org/10.3402/fnr.v60.29857.

11570'Halloran, F., Bruen, C., McGrath, B., Schellekens, H., Murray, B., Cryan, J. F., . . . Giblin, L.

1158 (2018). A casein hydrolysate increases GLP-1 secretion and reduces food intake. *Food*

1159 Chem, 252, 303-310. <u>https://doi.org/10.1016/j.foodchem.2018.01.107</u>.

- 1160Offret, C., Fliss, I., Bazinet, L., Marette, A., & Beaulieu, L. (2019). Identification of A Novel
 Antibacterial Peptide from Atlantic Mackerel belonging to the GAPDH-Related
 Antimicrobial Family and Its In Vitro Digestibility. *Marine drugs, 17*(7).
 https://doi.org/10.3390/md17070413.
- 1164Ono, S., Hosokawa, M., Miyashita, K., & Takahashi, K. (2003). Isolation of Peptides with
- 1165 Angiotensin I-converting Enzyme Inhibitory Effect Derived from Hydrolysate of Upstream
- 1166
 Chum Salmon Muscle.
 Journal of Food Science, 68(5), 1611-1614.

 1167
 https://doi.org/10.1111/j.1365-2621.2003.tb12300.x.
- 1168Ono, S., Hosokawa, M., Miyashita, K., & Takahashi, K. (2006). Inhibition properties of dipeptides
- from salmon muscle hydrolysate on angiotensin I-converting enzyme. International
- 1170
 Journal of Food Science & Technology, 41(4), 383-386.

 1171
 https://doi.org/10.1111/j.1365-2621.2005.01080.x.
- 1172Pak, V. V., Koo, M., Lee, N., Kim, M. S., & Kwon, D. Y. (2005). Structure—Activity
 Relationships of the Peptide Ile-Ala-Val-Pro and Its Derivatives Revealed Using the SemiEmpirical AM1 Method. *Chemistry of natural compounds*, *41*(4), 454-460.

1175Petricorena, Z. C. (2015). Chemical Composition of Fish and Fishery Products. In P. C. K. Cheung

- 1176 (Ed.), *Handbook of Food Chemistry* (pp. 1-28). Berlin, Heidelberg: Springer Berlin
 1177 Heidelberg.
- 1178Pezeshk, S., Ojagh, S. M., Rezaei, M., & Shabanpour, B. (2019). Fractionation of Protein
 Hydrolysates of Fish Waste Using Membrane Ultrafiltration: Investigation of Antibacterial

and Antioxidant Activities. *Probiotics Antimicrob Proteins*, 11(3), 1015-1022.
https://doi.org/10.1007/s12602-018-9483-y.

1182Picot, L., Bordenave, S., Didelot, S., Fruitier-Arnaudin, I., Sannier, F., Thorkelsson, G., . . . Piot,

1183 J. M. (2006). Antiproliferative activity of fish protein hydrolysates on human breast cancer

 1184
 cell
 lines.
 Process
 Biochemistry,
 41(5),
 1217-1222.

 1185
 https://doi.org/https://doi.org/10.1016/j.procbio.2005.11.024.

1186Provansal, M.M., Cuq, J.L. and Cheftel, J.C., 1975. Chemical and nutritional modifications of

1187 sunflower proteins due to alkaline processing. Formation of amino acid crosslinks and

1188 isomerization of lysine residues. *Journal of agricultural and food chemistry*, 23(5), pp.938-

1189 943. https://doi.org/10.1021/jf60201a030. https://doi.org/10.1021/jf60201a030

1190Qian, Z. J., Je, J. Y., & Kim, S. K. (2007). Antihypertensive effect of angiotensin i converting
enzyme-inhibitory peptide from hydrolysates of Bigeye tuna dark muscle, Thunnus obesus. *J Agric Food Chem*, 55(21), 8398-8403. <u>https://doi.org/10.1021/jf0710635</u>.

1193Rabiei, S., Rezaei, M., Asgharzade, S., Nikoo, M., & Rafieia-kopai, M. (2019). Antioxidant and
cytotoxic properties of protein hydrolysates obtained from enzymatic hydrolysis of
Klunzinger s mullet (Liza klunzingeri) muscle. *Brazilian Journal of Pharmaceutical*

1196 Sciences, 55. <u>http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1984-</u>
1197 82502019000100569&nrm=iso.

1198Reimer, R. A. (2006). Meat hydrolysate and essential amino acid-induced glucagon-like peptide1 secretion, in the human NCI-H716 enteroendocrine cell line, is regulated by extracellular
signal-regulated kinase1/2 and p38 mitogen-activated protein kinases. *J Endocrinol, 191*(1), 159-170. <u>https://doi.org/10.1677/joe.1.06557</u>.

1202Sabeena Farvin, K. H., Andersen, L. L., Nielsen, H. H., Jacobsen, C., Jakobsen, G., Johansson, I.,
1203 & Jessen, F. (2014). Antioxidant activity of Cod (Gadus morhua) protein hydrolysates: In

1204 vitro assays and evaluation in 5% fish oil-in-water emulsion. *Food Chemistry*, 149, 326-

1205 334. https://doi.org/https://doi.org/10.1016/j.foodchem.2013.03.075.

1206Salampessy, J., Phillips, M., Seneweera, S., & Kailasapathy, K. (2010). Release of antimicrobial

1207 peptides through bromelain hydrolysis of leatherjacket (Meuchenia sp.) insoluble proteins.

1208FoodChemistry,120(2),556-560.

1209 <u>https://doi.org/https://doi.org/10.1016/j.foodchem.2009.10.054</u>.

1210Samaranayaka, A. G., Kitts, D. D., & Li-Chan, E. C. (2010). Antioxidative and angiotensin-Iconverting enzyme inhibitory potential of a Pacific Hake (Merluccius productus) fish
protein hydrolysate subjected to simulated gastrointestinal digestion and Caco-2 cell
permeation. *J Agric Food Chem*, 58(3), 1535-1542. https://doi.org/10.1021/jf9033199.

1214Sangtanoo, P., Srimongkol, P., Saisavoey, T., Reamtong, O., & Karnchanatat, A. (2020). Anti-

1215 inflammatory action of two novel peptides derived from peanut worms (Sipunculus nudus)

1216 in lipopolysaccharide-induced RAW264.7 macrophages. Food & Function, 11(1), 552-

1217 560. <u>https://doi.org/10.1039/C9FO02178G</u>.

1218Segura-Campos, M., Chel-Guerrero, L., Betancur-Ancona, D., & Hernandez-Escalante, V. M.

1219 (2011). Bioavailability of Bioactive Peptides. *Food Reviews International*, 27(3), 213-226.

1220 https://doi.org/10.1080/87559129.2011.563395.

1221Seniman, M. S. M., Yusop, S. M., & Babji, A. S. (2014). Production of enzymatic protein

1222 hydrolysates from freshwater catfish (Clarias batrachus). *AIP Conference Proceedings*,

1223 *1614*(1), 323-328. <u>https://doi.org/10.1063/1.4895216</u>.

1224Sharkey, S. J., Harnedy-Rothwell, P. A., Allsopp, P. J., Hollywood, L. E., FitzGerald, R. J., &
O'Harte, F. P. M. (2020). A Narrative Review of the Anti-Hyperglycemic and Satiating
Effects of Fish Protein Hydrolysates and Their Bioactive Peptides. *Molecular Nutrition & Food Research, 64*(21), 2000403. <u>https://doi.org/https://doi.org/10.1002/mnfr.202000403</u>.

1228Shen, Q., Guo, R., Dai, Z., & Zhang, Y. (2012). Investigation of Enzymatic Hydrolysis Conditions
on the Properties of Protein Hydrolysate from Fish Muscle (Collichthys niveatus) and
Evaluation of Its Functional Properties. *Journal of Agricultural and Food Chemistry*,

1231 60(20), 5192-5198. <u>https://doi.org/10.1021/jf205258f</u>.

1232Song, R., Wei, R.B., Luo, H.Y. and Yang, Z.S. (2014). Isolation and identification of an

1233 antiproliferative peptide derived from heated products of peptic hydrolysates of half-fin

1234 anchovy (Setipinna taty). Journal of Functional Foods, 10, 104-111.
1235 https://doi.org/10.1016/j.jff.2014.06.010

1236Song, R., Wei, R., Zhang, B., Yang, Z. and Wang, D. (2011). Antioxidant and antiproliferative

1237 activities of heated sterilized pepsin hydrolysate derived from half-fin anchovy (Setipinna

1238 taty). *Marine Drugs*, 9(6), pp.1142-1156. <u>https://doi.org/10.3390/md9061142</u>

1239Suetsuna, K. and Osajima, K. (1986). The inhibitory activities against angiotensin I-converting
enzyme of basic peptides originating from sardine [Sardinops metanosticta] and hair tail
[Trichiurus lepturus] meat. *Bulletin of the Japanese Society of Scientific Fisheries, 52*(11),

1242 pp.1981-1984.

1243Suleria, H. A. R., Gobe, G., Masci, P., & Osborne, S. A. (2016). Marine bioactive compounds and
health promoting perspectives; innovation pathways for drug discovery. *Trends in Food Science* & *Technology*, 50, 44-55.
https://doi.org/https://doi.org/10.1016/j.tifs.2016.01.019.

1247Sung, N.-Y., Jung, P.-M., Yoon, M., Kim, J.-S., Choi, J.-i., Jeong, H. G., ... Kim, J.-H. (2012).

1248 Anti-inflammatory effect of sweetfish-derived protein and its enzymatichydrolysate on

1249 LPS-induced RAW264.7 cells via inhibition of NF-κB transcription. *Fisheries Science*,

1250 78(2), 381-390. https://doi.org/10.1007/s12562-011-0461-5.

1251Toopcham, T., Mes, J. J., Wichers, H. J., Roytrakul, S., & Yongsawatdigul, J. (2017).

1252 Bioavailability of angiotensin I-converting enzyme (ACE) inhibitory peptides derived from

1253 Virgibacillus halodenitrificans SK1-3-7 proteinases hydrolyzed tilapia muscle proteins.
1254 *Food Chem*, 220, 190-197. https://doi.org/10.1016/j.foodchem.2016.09.183.

1255Toopcham, T., Mes, J. J., Wichers, H. J., & Yongsawatdigul, J. (2017). Immunomodulatory

1256 activity of protein hydrolysates derived from Virgibacillus halodenitrificans SK1-3-7

1257 proteinase. *Food Chem, 224*, 320-328. <u>https://doi.org/10.1016/j.foodchem.2016.12.041</u>.

1258Tørris, C., Molin, M., & Småstuen, M. C. (2017). Lean fish consumption is associated with

beneficial changes in the metabolic syndrome components: a 13-year follow-up study from

1260 the Norwegian Tromsø study. *Nutrients*, 9(3), 247. <u>https://doi.org/10.3390/nu9030247</u>

1261

1262Tulipano, G., Sibilia, V., Caroli, A. M., & Cocchi, D. (2011). Whey proteins as source of dipeptidyl
dipeptidase IV (dipeptidyl peptidase-4) inhibitors. *Peptides*, 32(4), 835-838.
https://doi.org/10.1016/j.peptides.2011.01.002.

1265Valero, Y., Saraiva-Fraga, M., Costas, B. and Guardiola, F.A. (2020). Antimicrobial peptides from

fish: Beyond the fight against pathogens. *Reviews in Aquaculture*, 12(1), 224-253.
https://doi.org/10.1111/raq.12314

1268Vázquez, J. A., Blanco, M., Massa, A. E., Amado, I. R., & Pérez-Martín, R. I. (2017). Production 1269 of Fish Protein Hydrolysates from Scyliorhinus canicula Discards with Antihypertensive

1270 and Antioxidant Activities by Enzymatic Hydrolysis and Mathematical Optimization Using

1271ResponseSurfaceMethodology.MarineDrugs,15(10).1272https://doi.org/10.3390/md15100306.

1273Wang, B., Gong, Y.-D., Li, Z.-R., Yu, D., Chi, C.-F., & Ma, J.-Y. (2014). Isolation and
characterisation of five novel antioxidant peptides from ethanol-soluble proteins
hydrolysate of spotless smoothhound (Mustelus griseus) muscle. *Journal of Functional Foods*, 6, 176-185. https://doi.org/https://doi.org/10.1016/j.jff.2013.10.004.

1277Wang, S., Zeng, X., Yang, Q., & Qiao, S. (2016). Antimicrobial Peptides as Potential Alternatives

- to Antibiotics in Food Animal Industry. *International journal of molecular sciences*, 17(5).
 https://doi.org/10.3390/ijms17050603.
- 1280Wang, Y.-M., Xin, P., He, Y., Changfeng, C., & Bin, W. (2020). Hypolipidemic Activities of Two
- 1281 Pentapeptides (VIAPW and IRWWW) from Miiuy Croaker (Miichthys miiuy) Muscle on
- 1282 Lipid Accumulation in HepG2 Cells through Regulation of AMPK Pathway. *Applied*1283 *Sciences*, 10, 817.
- 1284White, U. A., & Stephens, J. M. (2010). Transcriptional factors that promote formation of white
 adipose tissue. *Mol Cell Endocrinol, 318*(1-2), 10-14.
 https://doi.org/10.1016/j.mce.2009.08.023.
- 1287Wijesekara, I., Qian, Z.-J., Ryu, B., Ngo, D.-H., & Kim, S.-K. (2011). Purification and
 identification of antihypertensive peptides from seaweed pipefish (Syngnathus schlegeli)
 muscle protein hydrolysate. *Food Research International, 44*(3), 703-707.
 https://doi.org/https://doi.org/10.1016/j.foodres.2010.12.022.
- 1291Wu, H., He, H.-L., Chen, X.-L., Sun, C.-Y., Zhang, Y.-Z., & Zhou, B.-C. (2008). Purification and
- identification of novel angiotensin-I-converting enzyme inhibitory peptides from shark
- 1293
 meat
 hydrolysate.
 Process
 Biochemistry,
 43(4),
 457-461.

 1294
 https://doi.org/https://doi.org/10.1016/j.procbio.2008.01.018.
- 1295Yokoyama, K., Chiba, H., & Yoshikawa, M. (1992). Peptide inhibitors for angiotensin Iconverting enzyme from thermolysin digest of dried bonito. *Biosci Biotechnol Biochem*,
- 1297 56(10), 1541-1545. <u>https://doi.org/10.1271/bbb.56.1541</u>.
- 1298Zhang, L., Zhang, S., Song, H., & Li, B. (2018). Effect of Collagen Hydrolysates from Silver Carp
- 1299 Skin (Hypophthalmichthys molitrix) on Osteoporosis in Chronologically Aged Mice:
- 1300 Increasing Bone Remodeling. *Nutrients, 10*(10). <u>https://doi.org/10.3390/nu10101434</u>.

1301Zhang, N., Zhang, C., Chen, Y., & Zheng, B. (2016). Purification and Characterization of
Antioxidant Peptides of Pseudosciaena crocea Protein Hydrolysates. *Molecules (Basel, Switzerland)*, 22(1), 57. https://doi.org/10.3390/molecules22010057.

1304Zhang, Q., Su, G., Zhao, T., Wang, S., Sun, B., Zheng, L., & Zhao, M. (2019). The memory

1305 improving effects of round scad (Decapterus maruadsi) hydrolysates on sleep deprivation-

1306 induced memory deficits in rats via antioxidant and neurotrophic pathways. Food &

1307 Function, 10(12), 7733-7744. <u>https://doi.org/10.1039/C9FO00855A</u>.

1308Zhang, X., Yang, F., Jiang, Q., Xu, Y., & Xia, W. (2018). Improvement of Antioxidant Activity

1309 of Grass Carp (Ctenopharyngodon idella) Protein Hydrolysate by Washing and Membrane

1310 Removal Pretreatments and Ultrasonic Treatment. Journal of Aquatic Food Product

1311 *Technology*, 27(5), 580-591. <u>https://doi.org/10.1080/10498850.2018.1461155</u>.

1312Zhang, Z., Hu, X., Lin, L., Ding, G., & Yu, F. (2019). Immunomodulatory Activity of Low

1313 Molecular-Weight Peptides from Nibea japonica in RAW264.7 Cells via NF-κB Pathway.

1314 *Marine drugs*, 17(7), 404. <u>https://doi.org/10.3390/md17070404</u>.

1315