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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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9

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.

24

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

26

27   **Abbreviations:** AA, amino acid; AAPH, 2,2-azobis-(2-amidino- propane) dihydrochloride;

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;

30   CCK, cholecystokinin; C/EBPα, CCATT/enhancer binding protein α; CFU, colony forming

31   unit, COX-2, cyclooxygenase-2; DH, degree of hydrolysis; DPPH, 2,2-diphenyl-1-

32   picrylhydrazyl; EC_{50}, 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; H_2O_2, 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; O_2^-, superoxide anion; ORAC,

40   oxygen radical absorbance capacity; OH, hydroxyl; PPARγ, peroxisome proliferator-activated

41   receptor γ; PGE_2, prostaglandin E_2; 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-α, tumour necrosis factor-α; ZPH, zebra blenny protein

46   hydrolysate.

47

1 Introduction

Fish is a rich source of protein, ranging from 10-25% depending on species, with 15-25% of total protein located in fish muscle (Petricorena, 2015). Fish muscle proteins can be divided into 3 groups, namely structural protein (approximately 70-80% of total protein content), 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 6.0%, Thr 4.6%, Met-Cys 4.0% and Val at 6.0% (Hayes & Flower, 2013). Marine organisms are reported to produce a variety of potent bioactive compounds as they are forced to live in a complex environment which is exposed to extreme conditions of salinity, pressure, temperature and illumination (Hamed et al., 2015). Bioactive peptides purified from fish sources have garnered considerable interest in recent times with potential applications in both food and pharmaceutical industries. Bioactive fish protein hydrolysates (FPH) and peptides are desirable functional food ingredients due to their natural availability, relatively low-cost extraction 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 (Suleria et al, 2016). The introduction of the 'landing obligation' policy by the European Commission in 2019 has maximized protein harvest from low-value fish species, thereby 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.

83   This paper will critically review current knowledge emerging from *in vitro* model systems

84   on the bioactive potential of protein hydrolysates and peptides isolated from various fish

85   muscle sources (Figure 1). We highlight limitations of studies, as well identify gaps in the

86   existing knowledge of bioactive fish peptides which has enabled us to recommend future

87   research opportunities.

88   **2   Production of FPH**

89   The bioactivity of food-derived protein hydrolysate ultimately depends on peptide and AA

90   composition. However, the composition of the resulting fraction is highly influenced by the

91   protein source, method of hydrolysis, hydrolysis conditions and degree of hydrolysis (DH).

92   Protein hydrolysates can be produced via (a) enzymatic hydrolysis with proteases sourced from

93   various commercial animal, microbial and plant sources, (b) fermentation with proteolytic

94   microorganisms, or (c) chemical hydrolysis with either alkali or acid. Chemical hydrolysis is

95   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;

98   Provansal et al., 1975). However, it does play a role in bioactive peptide release during

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.

109

110 2.1. Microbial enzymes

111   Microbial enzymes commonly used for the release of bioactive protein hydrolysates

112 from fish include Flavourzyme® (*Aspergillus oryzae*), 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

catfish (*C.batrachus*) protein to be more susceptible to hydrolysis with Alcalase® than plant-derived enzyme papain, demonstrating a direct correlation between DH and peptide content (Table 1). In contrast, Fonseca et al. (2016) reported Cobia *(Rachycentron canadum)* meat protein showed greater susceptibility to Protamex® hydrolysis exhibiting a DH value of 25.94% after 760 min compared to Alcalase® (10% after 300 min) and Flavourzyme® (12% after 420 min).

2.2. Animal-derived enzymes

Digestive enzymes from bovine and porcine GI tracts such as pepsin, trypsin, and chymotrypsin are also commonly used for production of biologically active protein hydrolysates from various fish sources (Bkhairia et al. 2016; Chi et al. 2014; Darewics et al., 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 muscle protein hydrolysate exhibited a higher DH value (49.12%) and subsequent ACE inhibitory activity than hydrolysates prepared with trypsin (DH 30.52%), or α-chymotrypsin (DH 28.75%). However, all rainbow trout muscle protein hydrolysates showed greater susceptibility to digestive proteases than Alcalase®, Neutrase®, or papain (Kim & Byun, 2012). Chi et al. (2014) reported that the most influential hydrolysis parameters on DH of trypsin-prepared monkfish protein hydrolysates were temperature, pH, enzyme/substrate (E/S) ratio and time, respectively. The maximum DH ($19.83 \pm 0.82\%$) of monkfish protein hydrolysate was obtained when hydrolysis conditions for trypsin were 40°C, pH 8.0, E/S 2% with 4 h hydrolysis period (Table 1). Under-utilised skipjack tuna (*Katsuwonus pelamis*) protein was highly susceptible to hydrolysis with either trypsin or Protamex® exhibiting DH values of 71.68% and 78.33%, respectively, however, trypsin was chosen for future hydrolysis of skipjack tuna protein due to its more attractive price point (Liu et al., 2015). A study by

145   Darewicz et al. (2014) compared *in vitro* and *ex vivo* hydrolysis of salmon myofibrillar and

146   sarcoplasmic proteins and reported *in vitro* hydrolysis with porcine pepsin or pepsin and

147   Corolase PP was more efficient than human gastric or gastric and duodenal juices, respectively,

148   as less intact protein was measured in *in vitro* hydrolysed fractions, indicating further

149   hydrolysis and production of small MW peptides. This study demonstrates the complexity of

150   the human digestive system and confirms that *in vitro* results may not always predict *in vivo*

151   results.

152

153   2.3. Plant-derived enzymes

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%,

157   respectively, compared to a DH of 13.3% for the hydrolysate produced with the microbial

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   2.3. Production challenges

166   The shortcomings associated with enzymatic hydrolysis of food proteins include solubility and

167   bitterness, both of which are highly influenced by DH, proteolytic enzyme and substrate

168   employed. Bitterness and solubility issues impose sensory and processing challenges,

169   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

172 less hydrophobic, bitter AA, Phe, than Alcalase® hydrolysis (Shen et al., 2012). Hydrophobic

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

176 (2004) reported that hydrolysates obtained by use of Alcalase® were more bitter than

177 hydrolysates obtained using Flavourzyme® as measured by sensory analysis. However, a

178 secondary treatment with Flavourzyme® did not further reduce bitterness of the fraction,

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

182 in MW, hydrophobicity and polar groups (Leni et al., 2020), Liu et al. (2015) reported

183 hydrolysis of under-utilised skipjack tuna protein for 5 h completely degraded small MW

184 peptides to AA and the highest soluble protein content (80%) was observed after 2.5 hr

185 hydrolysis with trypsin or Protamex. Glycation of the trypsin hydrolysate with alginate

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.

191

192

193   3   **Biological activity of fish muscle protein hydrolysates and peptides as reported *in***

194   ***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-α

202   (TNF-α), interleukin (IL)-6 and IL-1β and inflammatory mediators such as prostaglandin E_2

203   (PGE_2) 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

210   AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK   (MW:

211   6309.49 Da) (Table 2). The peptide (20 mg protein/mL) reduced NO production by 40.9 ±

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

AAs enhance attraction and reactivity with the cell membrane and possibly promote downstream signalling pathways with anti-inflammatory effects (Sangtanoo et al., 2020). In a previous study, Kangsanant et al. (2014) demonstrated that ultrasonic pre-treatment of tilapia protein hydrolysate prior to Flavourzyme® hydrolysis demonstrated superior NO inhibitory activity than non- pre-treated hydrolysates ($p<0.05$). It was suggested that ultrasonic pre-treatment promoted protein unfolding, thereby increasing enzyme accessibility to its cleavage sites. Anti-inflammatory protein hydrolysates purified from Argentine croaker were reported to be rich in AAs Glu, Asp, Lys, Leu, Arg, and Ala (Da Rocha et al., 2018). These hydrolysates were produced from Argentine croaker isolate and Argentine croaker myofibrillar protein with varying DH (DH: 10-20%) using either Alcalase® or Protamex® (Table 2). Interestingly, as DH increased from 10 to 20%, MW distribution decreased and the content of hydrophobic AAs increased ($p<0.05$). The hydrolysate produced from Argentine croaker myofibrillar via Protamex® hydrolysis with DH 10% at 5.0 mg/mL exhibited greater NO inhibitory activity in LPS- activated RAW264.7 cells (24h incubation) than all other hydrolysates tested ($p<0.05$) suggesting a role for peptides in its bioactivity rather than just free AA content. Hydrolysates prepared from Argentine croaker isolate and myofibrillar also demonstrated *in vitro* antioxidant activity and microbial-inhibitory activity in *Brochothrix thermosphacta, Listeria innocua*, and *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 Lys-Ile-Trp-His-His-Thr-Phe, Val-His-Tyr-Ala-Thr-Val-Asp-Tyr, and His-Leu-Asp-Asp-Ala-Leu-Arg-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 μM and 25 μM) 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 μg/ 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 PGE$_2$. 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)-$_k$B

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

266 individual fractions with various MWs; >10 kDa (SFTPH-I), 5-10 kD-0a (SFTPH-II) and <5

267 kDa (SFTPH-III). SFTPH-I (200 μg/ mL) significantly reduced production levels of

268 inflammation mediators NO and PGE_2 ($p < 0.05$) and pro-inflammatory cytokines IL-6, IL-1β

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

276 from *V.halodenitificans* SKI-3-7 significantly increased gene expression of IL-1β and COX-2

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-1β and activated the NF-κB signalling

283 pathway by upregulating NF-κB pathway-related proteins including IκB 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.

287 Although the majority of *in vitro* immunomodulatory studies focus on regulation of

288 pro-inflammatory cytokines IL-6 and TNF-α in LPS-stimulated macrophage, additional

289 possible mechanisms are elucidated in *in vivo* studies, including the promotion of natural killer

290 cells, stimulation of lymphocytes such as T cells and B cells, and stimulation of secretory

291 immunoglobulin A (S-IgA), thereby enhancing levels of mucosal immunity in the gut.

292 Additional clinical trials are necessary to understand the true effect of specific FPHs on the

293   immune system. A study on the immunomodulatory potential of FPHs in humans reported

294   salmon protein hydrolysate (Amizate) orally administrated (3 or 6g/ day, 4 months) to

295   malnourished Indian school children induced no effect on serum immunoglobulins IgG, IgM

296   or IgA or CD4/ CD8 lymphocyte ratio (Nesse et al., 2011).

297

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; IC_{50} 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 IC_{50} 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   (IC_{50} value of 0.61 mg/mL) compared with hydrolysates prepared with trypsin (IC_{50} value of

329   1.09 mg/mL) and α-chymotrypsin (IC_{50} 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   63.9 μM. 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 (IC_{50} values of 47.3 and 86.6 μg protein/mL, respectively) than pepsin plus

340   pancreatin hydrolysates (IC_{50} 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-

343 inhibitory activity, possibly due to the presence of ACE inhibiting di- to penta- peptides rich

344 in 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).

349 Furthermore, fractionation of ACE-inhibiting Pacific hake FPH prepared via autolysis (10^7 *K.*

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 <3

355 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 (IC_{50} 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

368 survive gut transit via simulated gastrointestinal digestion (SGID) suggesting ACE-inhibiting

369 ability will be maintained when administered orally (Balti et al., 2015; Chen et al., 2012;

370 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 IC_{50} 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).

386   The position of Trp residues is also important for ACE-inhibition and inhibition

387 mechanism of the peptides. Peptides with Trp at the C-terminal residue, namely Ala-Trp, Val-

388 Trp, Met-Trp, Ile-Trp, Leu-Trp with IC_{50} values of 6.4, 2.5, 9.8 and 17.4 μM, respectively,

389 showed non-competitive inhibition (Table 3). Whereas, reversed sequence peptides with Trp

390 at the N terminal including Trp-Ala, Trp-Leu, Trp-Met showed reduced ACE inhibitory

391 activity and acted via competitive inhibition (Ono et al., 2006). Similarly, Phe-Leu showed

392 non-competitive ACE inhibitory activity with an IC_{50} value of 13.6 μM. However, Leu-Phe

393 showed competitive ACE inhibitory activity with an IC$_{50}$ value of 383.3 µM. 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 IC$_{50}$ value of 1.2 µM.

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 (O$_2$) radical

414 scavenging activity. Other common non-cellular *in vitro* antioxidant assays include metal

415 chelating activity and ferric reducing antioxidant power (FRAP).

416   It is well known that protease and proteolysis conditions employed ultimately

417 determines the size and AA composition of the resulting fraction, and thereby, plays a crucial

418   role in the generation of antioxidant hydrolysates. Bashir et al. (2018) prepared a number of

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.

436   Bkhairia et al. (2016) also investigated the effects of various proteases on antioxidant

437   activity on golden grey mullet (*Liza aurata*) protein hydrolysates and expanded the study to

438   include AA analysis of resulting fractions. Golden grey mullet hydrolysates were prepared with

439   enzymatic preparations from *P. aeruginosa* A2 and *Bacillus subtilis* A26, crude enzyme from

440   *L. aurata*, trypsin or esperase and subsequent antioxidant assays deemed the hydrolysate

441   prepared with *B. subtilis* A26 proteases (rich in Glx, Gly, and Phe) exhibited the highest DPPH

442   and ABTS radical scavenging activity (IC_{50} 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)

444 induced the highest reducing power as determined by its ability to react with potassium

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

447 human erythrocytes, indicating their non-toxic effect and thereby, may be suitable for

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

458 to exhibit reduced radical scavenging activity and reducing power and higher Fe^{2+}-chelating

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

465 Alcalase® or Protamex® ($p<0.05$). Protamex® Argentine croaker myofibrillar protein

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-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 miiuy 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.

485 Low MW fractions from round scad protein hydrolysate (<5 kDa) and cod protein

486 hydrolysate (<3 kDa) exhibited higher radical scavenging activity and reducing power than

487 larger fractions (>10 kDa and >5 kDa, respectively) (Jiang et al., 2014; Sabeena Farvin et al.,

488 2014) (Table 4). Peptides His-Asp-His-Pro-Val-Cys and His-Glu-Lys-Val-Cys were purified

489 from the <5 kDa fraction of round scad muscle protein hydrolysate and effectively scavenged

490 DPPH radicals (EC_{50} values of 0.068 ± 0.001 and 0.031 ± 0.001 mM, respectively) and O_2^-

491 radicals (EC_{50} 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

500   superior antioxidant activity over His-Glu-Lys-Val-Cys (Jiang et al., 2014). Low MW fractions

501   (5-10, 1-3, and <1 kDa) rich in His residues were also purified from Alcalase[®] hydrolysed red

502   lionfish protein and demonstrated high antioxidant activity, with copper-chelating activity of

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).

506   Interestingly, goby FPH which exhibited significant antioxidant activity as measured

507   by DPPH radical scavenging activity, lipid peroxidation inhibition, β-carotene bleaching

508   inhibition, and metal chelating activity also effectively inhibited lipid peroxidation of turkey

509   meat sausage by 50% by storage day 3 compared with the control (Nasri et al., 2013). As a

510   result, it is possible that incorporation of goby protein hydrolysate as powder with turkey meat

511   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 μM) 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 H_2O_2-

518   treated SHSY5Y was also reduced dose-dependently when exposed to LPHs (0.37–0.73

519   mg/mL) for 24h as measured by agarose gel electrophoresis (Table 4). Tripeptide Phe-Tyr-Tyr

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 ± 4.5% compared with cells treated with AAPH alone

531   (Altinelataman et al., 2019). In the same study, Alcalase® hydrolysed gilthead seabream

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 H_2O_2-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 H_2O_2-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

551 identified via *in vitro* testing, very few studies exist investigating the antioxidant activity of

552 fish muscle protein hydrolysates *in vivo* (Nazeer, Kumar, & Ganesh, 2012, Bashir et al., 2018).

553

554 **3.4   Anti-microbial activity**

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

23

(GCB) sorbent for purification of medium-sized peptide and short-sized peptide fractions, respectively (Cerrato et al., 2020). The C18 digested fraction exhibited greater antibacterial activity against *S. aureus* bacteria with a minimum inhibition concentration (MIC) value of 1.0 ± 0.1 mg/mL than the GCB fraction (MIC value of 3.5 ± 0.1 mg/mL) (Table 5). Although a total of 403 peptides from medium-sized peptide fraction and 572 peptides from the short-sized peptide fraction were identified, none of these peptides pre-existed on BIOPEP or PeptideDB databases. Interestingly, medium-size peptides were mostly hydrophilic with intermediate polarity and small-sized peptide fractions were composed of mainly hydrophobic, less anionic peptides, however, the combination of several anti-microbial peptides in the fraction may induce a synergistic effect, inhibiting *S. aureus* activity more effectively than a single peptide. Fraction 12 (MW not reported) purified from a bromelain hydrolysate of leatherjacket (*Meuchenia* sp.) muscle protein by size using a RP-HPLC C-18 preparative column demonstrated antimicrobial activities against gram-positive bacteria *Bacillus cereus* and *S. aureus* with a MIC of 4.3 mg/mL (Table 5) (Salampessy et al., 2010). A study by Da Rocha et 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 hydrolysates produced with either Alcalase® or Protamex® with DH 10% or 20%, all inhibited gram-positive bacteria *B. thermosphacta, L. innocua*, and *S. aureus*. However, only Alcalase® hydrolysates inhibited gram positive bacteria *Listeria monocytogenes*, and gram-negative bacteria *Yersinia enterecolitica*. Alcalase® treated hydrolysates had a lower MW distribution (<1285 Da), a higher content of hydrophobic AAs and as a result, more pronounced inhibition zones than Protamex® treated hydrolysates. Alcalase® hydrolysates dose-dependently increased inhibition zones in *Aeromonas hydrophila, B. thermospacta, Debaryomysces hanseii,* and *L. innocua* (1.25-7.5 mg/mL), with Alcalase® hydrolysed Argentine croaker 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

625 protease reduced cell viability in the human colon adenocarcinoma cell line, HT-29, by $39.6 \pm$

626 12.8% (Altinelataman et al., 2019). Similar antiproliferative activity was observed for blue

627 whiting protein hydrolysate (BWPH) (1mg/ mL) produced via hydrolysis with either

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 IC_{50} values of 1731.4 and 2922.5 μg/mL for AGS cells and

638 HeLa cells, respectively. The Alcalase® hydrolysate was subsequently fractionated with the

639 resulting low MW fraction (fraction F_2: 3.6 kDa, rich in hydrophobic AAs (78.1%)) inhibiting

640 cell growth of AGS, DLD-1 and HeLa cells with IC_{50} values of 577.1, 1163.3, and 887.2 μg/

641 mL, respectively (Jumeri & Kim, 2011). It is possible that low MW peptides have enhanced

interactions with cancer cell components via enhanced cell mobility and diffusivity than large

MW components, thereby improving anti-cancer activity. Song and colleagues (2011) also

reported that thermal treatment (121°C, 30 min) of pepsin hydrolysate derived from half-fin

anchovy increased free AAs Val, Leu, Phe, His and Arg, increased the number of peptides with

MW distribution of 3000-5000 Da and <300 Da ($p<0.005$), and ultimately, increased anti-

proliferative activity against DU-145 human prostate cancer cells, 1299 human lung cancer

cells, and 109 human oesophagus cancer cells ($p<0.05$) compared to the non-heat sterilised

hydrolysate.

Hydrophobic AA residues are essential for the formation of a hydrophobic tail in the

COOH-terminal region, an attribute important for anti-cancer peptides. A recent review

discusses the classifications and structure-activity relationship of anti-cancer peptides in more

detail (Chiangjong et al., 2020). In short, in contrast to healthy cells, cancer cells possess

phosphatidylserine, a negatively charged phospholipid, allowing for electrostatic attraction

between cationic peptides and cancer cells. Upon electrostatic interaction with the membrane

surface of cancer cells, peptides arrange in either an α-helix or β-sheet, resulting in cell

membrane disruption. Anti-cancer peptides can be classified as i) molecularly targeted

peptides, ii) binding peptides, or iii) cell-stimulating peptides. Hydrophobic, positively charged

Lys- and Arg-rich peptides can induce cancer cell toxicity via disruption and penetration of

anionic, hydrophobic cancer cell membranes; a mechanism known as 'snorkeling'. The peptide

Tyr-Ala-Leu-Pro-Ala-His was subsequently purified from the heat-treated pepsin hydrolysate

of half-fin anchovy. Although this peptide inhibited prostate cancer PC-3 cells by 50% at 11.4

mg/mL, modification of the peptide to Tyr-Ala-Leu-Arg-Ala-His improved its inhibitory

activity (IC_{50} value of 8.1 mg/mL). The enhanced cell permeation efficacy of Arg-rich peptides

may be due to the hydrogen-bond formation of guanidine moiety in Arg with phosphates,

sulfates, and carboxylates on cellular components (Song et al., 2014). Peptides Leu-Pro-His-

667 Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr and Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr

668 purified from tuna dark muscle byproduct hydrolysates prepared with papain and Protease

669 XXIII induced a dose-dependent anti-proliferative effect on MCF-7 cells with IC_{50} values of

670 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

676 rich in Glu, Lys, Gly, and Thr induced a dose-dependent cytotoxic effect in the human

677 hepatoblastoma cell line, HepG2, with IC_{50} 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

683 challenges include conjugation of therapeutic peptides with cell-penetrating peptides to

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

687 combination of peptides with each or other anti-cancer drugs may also promote improve

688 efficacy and efficiency of chemotherapy.

3.6 Anti-obesity potential

Although enlargement of adipocytes is the main mechanism of weight gain in adults, obese people generally have a higher amount of adipocytes than non-obese people. However, adipocyte number is altered during childhood and adolescence and remains constant during adulthood in both obese and lean people (Spalding et al., 2008). Adipogenesis is regulated by various transcription factors including peroxisome proliferator-activated receptor γ (PPARγ), sterol regulatory element binding protein (SREBP)-1 and CCATT/enhancer binding protein α (C/EBPα) (White & Stephans, 2010). After a thorough search of the relevant literature, no adipocyte-modulating hydrolysate from a fish source was found. However, a peptide derived from desalinated boiled tuna extract (Asp-Ile-Val-Asp-Lys-Ile-Glu-Ile) (5 mg/mL) reduced triglyceride accumulation significantly ($p < 0.05$) in differentiated adipocytes (3T3-L1 cell line) compared with differentiated cells treated with media alone (Kim, Choi, Lee, & Nam, 2015). This peptide also reduced expression levels of C-EBPα and PPARγ, and expression levels of adipogenic and lipogenic genes in differentiated 3T3-L1 cells. Pentapeptides Val-Ile-Asp-Pro-Trp and Ile-Arg-Trp-Trp-Trp (100 μM) purified from papain hydrolysed miiuy croaker muscle (pH 7.5, 50°C, enzyme dose of 1.5%, 5h) significantly reduced oleic acid-induced lipid accumulation in human liver carcinoma cells (HepG2) after 24h exposure ($p < 0.05$ and $p < 0.01$, respectively) compared to the oleic acid model control (Wang et al., 2020). These pentapeptides (100 μM) also reduced intracellular triglyceride levels ($p < 0.01$ and $p < 0.001$, respectively), total cholesterol levels ($p < 0.01$ and $p < 0.001$, respectively), expression of lipogenesis genes (SREBP-1c, SREBP-2, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and 3-hydroxy-3-methyl-glutaryl-coenzyme-A reductase (HMGR)) and increased the expression levels of lipolysis genes (PPARα, acyl-CoA oxidase 1 (ACOX-1), and carnitine palmitoyltransferase-1 (CPT-1)) compared with cellular oleic acid model control. A database 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

717 stabilisation of a 'turn' conformation and formation of hydrogen bonds to the binding site of

718 HMGR, a known rate limiting enzyme in cholesterol biosynthesis. Moreover, peptide

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 anti-

763 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

769 a 13-year follow-up period (1994-1995, $n = 23,907$ to 2007–2008, $n = 12,981$) revealed that

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

784 (25 kg/m^2 ≤ body mass index (BMI) < 30 kg/m^2) adults over 90 days. BWPH is now

785 commercialised and marketed as Slimpro® (Nobile et al., 2016). In addition, peptides purified

786 from dried bonito (*katsuobushi*) via thermolysin digestion exhibiting ACE-inhibitory activities

787 *in vitro* were also shown to exhibit anti-hypertensive effects in spontaneously hypertensive rats

788 and borderline (high normal) and mildly hypertensive adults (1.4 g/ day orally administrated

789 over 5 weeks) (Fujita et al., 2001; Yokoyama et al., 1992). *Katsuobushi* oligopeptide received

790 official approval as Foods for Specific Health Use (FoSHU) in 1999 by the Ministry of Health

791 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.

800   Although bioactive fish hydrolysates and peptides are generally not as potent as

801 synthetic drugs, they could provide a safe and natural alternative for the prevention more than

802 the treatment of disease. However, for bioactive efficacy in a functional food offering, fish

803 hydrolysates similar to other food hydrolysates must overcome several hurdles not least of

804 which includes issues of processing, food formulation, sensory acceptance, survival during GI

805 digestion and bioavailability.

806   A systematic approach for optimization of the numerous parameters which influence

807 the production of bioactive peptides is now advised compared to 'one factor at a time' or 'trial

808 and error' approaches which should now be deemed obsolete (Chakrabarti et al., 2018). The

809 development of bioinformatics analysis in recent years has promoted a highly useful approach

810 for the generation of bioactive peptides via the utilization of computational data to predict

811 peptide sequences likely to induce specific bioactivities and elucidate structure-function

812 relationships. Peptide databases should be exploited to save time and expenses involved in

813 purifying fish peptides and testing various potential bioactivities. These databases also provide

814 information about peptide structure-function relationships, molecular docking, and peptide-

815 receptor interactions, which are essential for the development of therapeutic products.

816   Although *in vitro* investigations offer great insight into the potential bioactivities of

817 FPH, for FPHs to have use as bioactive agents with the aim to improve human health, more

818 clinical trials are required in order to determine FPH bioavailability and absorption through the

819 GI barrier ensuring eventual contact with target cells. In addition, future studies should

820 elucidate mechanisms of action of bioactive fish muscle protein hydrolysates, as well as,

821 identify individual bioactive peptides from fish protein fractions so that peptide structure-

822 function relationships can be further understood.

823

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)

846   This review offers a comparison of studies investigating the cellular *in vitro* bioactivity

847   of fish muscle protein hydrolysates and has collated numerous data informing the reader of

848   suitable protein sources, enzymes, and processing conditions for the generation of bioactive

849   hydrolysates. This review thereby may be a useful data base when designing future studies on

850   fish muscle protein hydrolysates/peptides *in vitro* or *in vivo* as no database listing exclusively

851   bioactive peptides derived from fish sources currently exists.

852   Overall, ACE inhibition appears to be the most promising bioactivity of fish muscle

853   protein hydrolysates/ peptides of the six bioactivities discussed in this review. Although

854   hydrolysates from other protein sources such as milk, meat and plant show similar inhibitory

855   activity, fish is now an abundant source of protein due to the 'landing obligation' policy which

856   was introduced by the European Commission with the aim of progressive elimination of

857   discards in all EU fisheries. Fish protein now represents an affordable alternative to milk

858   proteins which are currently the main source of bioactive peptides.

865   **Declaration of Competing Interest**

866   The authors declare no conflict of interest

867

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869

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