Assessment of the biological activity of fish muscle protein hydrolysates using *in vitro* model systems

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Abstract

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

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

Abbreviations: AA, amino acid; AAPH, 2,2-azobis-(2-amidino- propane) dihydrochloride; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; ACE, angiotensin-1-converting enzyme; BMI, body mass index; BWPH; blue whiting protein hydrolysate; CAT, catalase; CCK, cholecystokinin; C/EBPα, CCATT/enhancer binding protein α; CFU, colony forming unit, COX-2, cyclooxygenase-2; DH, degree of hydrolysis; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EC_{50}, concentration corresponding to half-maximal activity; E/S ratio, enzyme/ substrate ratio, FPH, fish protein hydrolysates; FOSHU, Foods for Specific Health Use; FRAP, ferric reducing antioxidant power; GCB, graphitized carbon black; GI, gastrointestinal; GLP-1, glucagon-like peptide-1; GSH, glutathione; GSH-Px, glutathione peroxidase; GPH, goby protein hydrolysate; H_2O_2, hydrogen peroxide; IL, interleukin; LPH, lanternfish protein hydrolysates; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MIC, minimum inhibition concentration; MW, molecular weight; NF, nuclear factor; NO, nitric oxide; O_2^-, superoxide anion; ORAC, oxygen radical absorbance capacity; OH, hydroxyl; PPARγ, peroxisome proliferator-activated receptor γ; PGE_2, prostaglandin E_2; PYY, peptide YY; RPH, ray protein hydrolysate; ROS, reactive oxygen species; RP-HPLC, reversed-phase high-performance liquid chromatography; RSM, response surface methodology; SGID, simulated gastrointestinal digestion; SOD, superoxide dismutase; SPH, sardinella protein hydrolysate; SREBP-1, sterol regulatory element binding protein; TNF-α, tumour necrosis factor-α; ZPH, zebra blenny protein hydrolysate.

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.

In vitro testing is often utilized for preliminary research, prior to testing via *in vivo* model systems, due to its cost-efficiency and ability to yield rapid and reproducible data. Although non-cellular bioassays are used to investigate some bioactivities including angiotensin-1-converting enzyme (ACE) inhibition and anti-microbial activity, cellular model systems are useful for investigating various bioactivities as well as unravelling the biological pathways activated upon contact of the bioactive compound with the target cells. Although *in vitro* studies provide a controlled environment for experimentation, cellular bioassays involve maintaining cells outside of the living organism; therefore, results must be interpreted carefully due to the innate complexity of organ systems *in vivo* (Jain et al., 2018). Bioactive FPH for oral consumption face the challenge of surviving the hydrolytic conditions of the GI tract, so 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.

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;
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

121 catfish (*C.batrachus*) protein to be more susceptible to hydrolysis with Alcalase® than plant-
122 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 2.2. Animal-derived enzymes

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
132 al., 2012). DH values of bioactive hydrolysates were not always reported. Peptic rainbow trout
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.

2.3. Plant-derived enzymes

Some studies found plant-derived proteases induced a higher DH in FPHs than alternatively sourced proteolytic enzymes. Catfish (*Pangasius hypothalamus*) meat protein 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 enzyme Neutrase® (Table 1) (Ha et al., 2017). Hydrolysis with bromelain increased the free AA content most effectively from 28.00 g/kg protein to 58.02 g/kg protein compared with papain and Neutrase® hydrolysis, however, bromelain hydrolysis had no effect on the antioxidant activity of catfish protein. Both papain and Neutrase® hydrolysates demonstrated increased radical scavenging activity compared with the non-hydrolysed control, potentially owing to the reduced hydrolysis of bioactive peptides.

2.3. Production challenges

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.

Hydrolysis of *Collichthys niveatus* protein with Neutrase® generated a hydrolysate with a 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 peptides are associated with bitter taste due to the presence of two functional units, the binding unit and the stimulating unit, responsible for binding with the bitter taste receptor and 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 hydrolysates obtained using Flavourzyme® as measured by sensory analysis. However, a secondary treatment with Flavourzyme® did not further reduce bitterness of the fraction, whereas, treatment of the FPH with n-butanol and cholestyramine resin did indeed reduce bitterness via extraction of bitter bile compounds (Dauksas et al., 2004).

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 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 hydrolysis with trypsin or Protamex. Glycation of the trypsin hydrolysate with alginate significantly improved solubility ($p<0.05$) compared with the non-glycated fraction in the pH range of 2-10 possibly due to the glycation-induced shift of isoelectric point towards a more acidic pH. It is well known that hydrolysates generally show low solubility at their isoelectric points, therefore it is probable that the basic pH of the trypsin hydrolysate influenced its solubility.

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

3.1 Inflammation modulatory activity

Table 2 details studies investigating the ability of fish muscle hydrolysates to modulate signals within inflammatory response pathways. The human body initiates inflammation in response to various stimuli including infections, injury, and toxins in an attempt to heal itself. Activation of macrophages is essential for initiation and continuation of defensive reactions as macrophages release various pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β and inflammatory mediators such as prostaglandin E_2 (PGE_2) and nitric oxide (NO) which improve tissue repair (Je & Kim, 2012). However, prolonged or excessive inflammation is associated with a wide range of diseases, including chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, and cancer.

Kangsanant et al. (2015) identified a novel anti-inflammatory peptide from Flavourzyme® hydrolysed tilapia muscle protein via gel filtration chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC) with an AA sequence of AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK (MW: 6309.49 Da) (Table 2). The peptide (20 mg protein/mL) reduced NO production by 40.9 ± 0.2% in the murine macrophage cell line RAW264.7 stimulated with lipopolysaccharide (LPS) for 48h; which was 100 fold higher than that of the crude hydrolysate. The presence of hydrophobic AAs residues in the purified peptide, namely, Ala, Phe, Leu, Val and Ile was hypothesized to play a significant role in its NO-inhibitory activity. Although further explanation regarding the relationship between hydrophobic AAs and their function in 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

inflammation mediators NO and PGE_2 ($p<0.05$) and pro-inflammatory cytokines IL-6, IL-1β and TNF-α ($p<0.01$) after 24h exposure to LPS-challenged RAW264.7 compared with macrophage exposed to LPS alone. It was determined that SFTPH-I inhibited production of pro-inflammatory mediators via reducing protein expression levels of iNOS and COX-2 and attenuating phosphorylation of MAPKs (ERK, p38 and JNK) in activated macrophages.

FPHs have also demonstrated pro-inflammatory activity *in vitro*. Activation of pro-inflammatory cytokines can enhance host defence against infection in immunodeficiency 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 in the human monocyte leukaemia cell line THP-1 stimulated with LPS, after 6h incubation ($p<0.05$) (Toopcham et al., 2017) (Table 2). Similarly, a low MW fraction (<1 kDa), labelled NJP, isolated from papain hydrolysed *Nibea Japonica* protein (200 μg/ mL) significantly upregulated protein expression of iNOS and production of NO in LPS-activated RAW264.7 cells ($p<0.01$) (Zhang, Hu, et al., 2019). At this concentration, it also increased production of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β and activated the NF-κB signalling pathway by upregulating NF-κB pathway-related proteins including IκB kinase (Ikk)-α and IKK-β. The neutral red internalization model was employed to demonstrate that NJP increased phagocytosis rate in RAW264.7 cells in a concentration-dependent manner; this initiated the 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

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

showed competitive ACE inhibitory activity with an IC_{50} value of 383.3 μM. A study by Enari et al. (2008) purified 20 active di- and tripeptides from salmon muscle papain hydrolysate and demonstrated the strongest ACE inhibition by Ile-Trp with an IC_{50} value of 1.2 μM.

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

3.3 Antioxidant activity

Antioxidant FPHs serve as valuable ingredients in functional foods considering their ability to extend shelf-life, as well as induce health benefits via promoting cellular redox balance. Several studies reported the antioxidant activity of FPHs and their ability to modulate oxidative stress pathways *in vitro* (Table 4). Oxidative stress is associated with many diseases including cancer, diabetes, rheumatoid arthritis, chronic inflammation, and numerous neurodegenerative diseases (Kumar et al., 2017). The antioxidant activity of marine hydrolysates and peptides has mainly been assessed via scavenging activity of free radicals and ROS in non-cellular *in vitro* assays, namely, 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), oxygen radical absorbance capacity (ORAC), hydroxyl (OH) radical scavenging activity and superoxide anion (O_2) radical scavenging activity. Other common non-cellular *in vitro* antioxidant assays include metal chelating activity and ferric reducing antioxidant power (FRAP).

It is well known that protease and proteolysis conditions employed ultimately 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.,

2014). The antioxidative activities of the peptides were proposed to be enhanced by the participation of hydrophobic AAs and one or more residues of His, Pro and Cys. The imidazole group and thiol group of His and Cys residues, respectively, promotes proton donation, thereby stabilising ROS. In particular, Cys is one of 3 AA in glutathione (GSH), a potent endogenous antioxidant in mammalian cells. Hydrolysates that are rich in Cys are likely to boost GSH cellular pathways. It is possible that antioxidant activity not only depends on the presence of a specific AA but also its quantity and position within the peptide sequence. The presence of His 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 (5-10, 1-3, and <1 kDa) rich in His residues were also purified from Alcalase® hydrolysed red lionfish protein and demonstrated high antioxidant activity, with copper-chelating activity of approximately 88%, as well as inhibiting hydrophobic β-carotene discolouration by 80% versus the negative control. His residues are indeed reported to have a strong binding affinity 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.

FPHs have also been shown to enhance antioxidant defence systems in various cell model systems. Protease N hydrolysed lanternfish protein hydrolysates (LPH) were demonstrated to prevent hydrogen peroxide (H_2O_2)-induced oxidative cell damage in human neuroblastoma cells (SHSY5Y) (Chai et al., 2013). MTT assay demonstrated that the viability 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

(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 ±
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

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 anti-
647 proliferative activity against DU-145 human prostate cancer cells, 1299 human lung cancer
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 (IC_{50} 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-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.

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 (PPARγ),
695 sterol regulatory element binding protein (SREBP)-1 and CCATT/enhancer binding protein α
696 (C/EBPα) (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-EBPα and PPARγ, 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-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 (PPARα, 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
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 ± 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 ± 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-

obesity potential of FPHs to other protein sources due to the methodological variation between studies i.e., cell type, cell density, exposure time, hydrolysate concentration. However, a recent review by Sharkey et al. (2020) concluded that many FPHs have potential to reduce body weight and improve body composition *in vivo* and in clinical studies. The Norwegian Tromsø Study is an epidemiological study with the focus of investigating the relationship between fish 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 consumption of lean fish once a week or more was associated with decreased future metabolic score, decreased triglyceride content, and increased high-density lipoprotein-cholesterol, whereas decreased waist circumference and blood pressure was identified only for men (Tørris et al., 2017).

4 Applications, challenges and future perspectives

FPHs represent desirable functional food ingredients owing to their beneficial impact on both health and food quality. Addition of antioxidant or anti-microbial FPHs to a food system may inhibit lipid peroxidation or growth of food spoilage microorganisms, respectively, thereby potentially extending shelf life of supplemented products. Not only do FPH have use as preservative ingredients, but some FPH are also commercialised functional food ingredients with health promoting claims. BWPH which induced CCK and GLP-1 secretion in STC-1 cells, was subsequently demonstrated to increase plasma concentrations of CCK and GLP-1, improve body composition and reduce body weight upon oral administration (1.4 g) to 120 overweight (25 kg/m^2 ≤ body mass index (BMI) < 30 kg/m^2) adults over 90 days. BWPH is now commercialised and marketed as Slimpro® (Nobile et al., 2016). In addition, peptides purified 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

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)

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.

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Declaration of Competing Interest

The authors declare no conflict of interest

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