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Antioxidant and Antihypertensive Protein Hydrolysates in Fish Products – a Review

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Abstract

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Fish proteins are a good source of bioactive peptides (BAPs). Such BAPs are derived through enzymatic hydrolysis of food proteins and can potentially by applied as health-promoting factors against chronic non-communicable diseases (NCDs), including arterial hypertension, cardiovascular disease and obesity. Antihypertensive and antioxidant BAPs derived from fish could represent a good alternative to synthetic drugs. This article reviews the literature on BAPs derived from fish and fish products, with an emphasis on antihypertensive and antioxidant properties and the impact of technological processes on the activity of BAPs. The review shows that BAPs isolated from fish exhibit quite good stability when applied under moderate physical conditions and after simulated *in vitro* digestion. Processing can increase the susceptibility of peptides to digestion in the digestive tract as well as improving absorption and immune system responses. Therefore, it is important to determine the optimal conditions under which proteins (and peptides) can be processed in order to maintain their bioactivity. Future research efforts on BAPs should be directed towards an elucidation of their activity after technological processes.

Keywords: bioactivity; functional food; fish protein hydrolysates; marine bioactive peptides; stability; technological process

Global fish consumption and the fish industry in general exhibit an upward trend (Benhabiles *et al.* 2012). Over the past 6 years, fish production has risen annually by 0.87% and fish consumption by almost 0.9% *per capita* (FAO 2016). One of the reasons for the increased interest of consumers in fish and fish products is the currently dominant global trend toward leading a healthy lifestyle and eating healthy food. Fish consumption fits perfectly in this trend, as fish are natural functional foods (Halldorsdottir *et al.* 2014). Fish meat, in addition to the desired lipid profile (Rombensoa *et al.* 2016), is also characterised by a composition of amino acids (AA) which is beneficial in biological and nutritional terms. This promotes the use of substances naturally occurring in fish in the pharmaceutical, cosmetic, medical and food industries (Ferraro *et al.* 2013; Lemes *et al.* 2016).

A protein precursor contained in fish is biologically inactive. Only its decomposition/degradation as a result of the activity of digestive enzymes (*in vivo*) or hydrolysis (*in vitro*) gives biologically active peptides (Erdmann *et al.* 2008; Möller *et al.* 2008). As a result of chemical and enzymatic hydrolysis, peptides of different sizes are formed, so-called fish protein hydrolysates (FPH) (Skanderby 1994). Enzymatic hydrolysis is the most common method of protein breakdown *in vitro*. In order to obtain FPH, the following enzymes are used for proteolysis: animal proteases – pepsin, trypsin; vegetable proteases – phytin, papain; and microbial enzymes – proteinase K, collagenase (Darewicz *et al.* 2015a).

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Chemical hydrolysis (using acids and bases) is used to produce FPH on an industrial scale. However, FPHs obtained as a result of enzymatic hydrolysis are characterised by better functionality and higher nutritional value than those obtained by chemical hydrolysis (Lafarga & Hayes 2016).

FPHs are an excellent source of bioactive peptides (BAPs), which influence the human organism biologically and physiologically (Rasika *et al.* 2013). They can regulate biological processes occurring in the body or act as neurotransmitters. The complexity of their functions results from the fact that they can act in the digestive tract, either in the intestinal epithelium or after absorption into the circulatory system (Darewicz *et al.* 2011).

BAPs are 2–20 AAs in size. A single residue never by itself constitutes a BAP. Rather, a minimum of two AAs are connected by a peptide bond, and such peptides have health benefits when consumed (EFSA Scientific Report 2009). The biological activity of BAPs is correlated with the sequence and composition of AAs in the protein (Mora *et al.* 2014; Singh *et al.* 2014). BAPs often contain hydrophobic AAs (Di Bernardini *et al.* 2011), usually proline, lysine and arginine (Rao 1991). The use of FPH as a source of BAPs on an industrial scale has many limitations related to the use of extreme conditions to obtain FPH. This is mainly the case when the product is treated with strong acids or bases (chemical hydrolysis).

Therefore, the purpose of this review article is to highlight current knowledge on the occurrence of antihypertensive and antioxidant peptides in different species of fish and the stability of BAPs. There is no clear understanding of how FPH generated through technological processing behave in primary products to which they are added. The relationship between the AA composition and the activity of BAPs derived from fish also remains to be fully elucidated.

Antihypertensive and antioxidant properties of BAPs

Angiotensin-converting enzyme (ACE) inhibitors are used in the treatment of hypertension and their efficacy is well documented (Lafarga & Hayes 2016). Natural ACE inhibitors are safer than synthetic medicines that reduce blood pressure, and their long-term use is not associated with any side effects (Kim & Wijesekara 2010). They are present in many food products, such as fish (Rousseau-Ralliard *et al.* 2010).

It is well documented that ACE inhibitors usually consist of a short AA sequence (He *et al.* 2013). In the sequence of ACE inhibitors, the following AAs dominate: tyrosine (Tyr), phenyloalanine (Phe), tryptophan (Trp), lysine (Lys), leucine (Leu), isoleucine (Ile), valine (Val) and arginine (Arg) (Murray & FitzGerald 2007). The peptides which are most effective are those containing hydrophobic AAs, mainly proline (Pro) in the C-terminal position and positively charged AAs (Arg and Lys) in the terminal position (Lemes *et al.* 2016). The prevalence of the above-mentioned AAs in ACE-inhibiting peptides was confirmed by research into structure-activity relationships (QSAR) (Wu *et al.* 2006).

Lassoued *et al.* (2015) reported that the antioxidant activity of BAPs is associated with the composition, sequence and hydrophobicity of AAs. Low molecular weights (0.5 and 1.5 kDa) and short chains of AAs (5–16 AA) correlate with strong antioxidant effect and promote fast absorption of a peptide in the intestine (Li & Yu 2015). A strong correlation with the antioxidant properties of the peptides is shown by hydrophobic and aromatic AA (Cheison *et al.* 2007). When present in a BAP, Tyr delivers a proton to suppress free radicals (Wang *et al.* 2008b). With its antioxidant properties, histidine (His) promotes chelation of metal ions, inactivation of active oxygen and scavenging of free radicals (Saito *et al.* 2003). His, Pro, cysteine (Cys), Tyr, Trp, Phe, and methionine (Met) delay lipid peroxidation, thus producing an antioxidant effect (Li & Yu 2015). We should bear in mind that the antioxidant effect of a single AA is far weaker than the effect of many AAs working together in the sequence of a BAP (Zhu *et al.* 2012).

Antihypertensive and antioxidant properties of BAPs in selected species of fish

BAPs in fish are produced by enzymatic or chemical hydrolysis and by the action of gastrointestinal proteases (Mora *et al.* 2017). The antioxidant and antihypertensive properties of BAPs occurring in popular European fish species are discussed below.

Carp (*Cyprinus carpio* L.) constitutes a valuable source of BAPs (Darewicz *et al.* 2016). Hydrolysis *ex vivo* using human digestive juices and *in vitro* using porcine digestive enzymes on carp muscle protein was studied by Borawska *et al.* (2016a). Myofibrillar (MP) and sarcoplasmic carp proteins (SP) showed a greater resistance to digestion by the

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porcine enzymes than by the human enzymes. All isolated peptides have demonstrated the ability to scavenge free radicals and to reduce the degree of iron oxidation. After the chewing phase/before hydrolysis, carp protein extracts showed higher ability to scavenge DPPH (e.g., MP hydrolysates before *in vitro* hydrolysis: 129 μM/g) than their *in vitro* and *ex vivo* hydrolysates (e.g., *in vitro* MP hydrolysates: 30 μM/g). In contrast, the ABTS scavenging ability increased during both hydrolysis reactions (e.g., MP hydrolysates before *in vitro* hydrolysis: 30.9 μM/g; *in vitro* MP hydrolysates: 232.3 μM/g). Using *in silico* analyses, the authors identified five peptides with antioxidant properties: FIKK, HL, IY, PW, and VY. A study on the same material and with the same research scheme was conducted by Darewicz *et al.* (2016), although they analysed the antihypertensive properties. The peptides IVY, IY, VY, ALPHA, and VKAGF with ACE inhibitory activity were common to both types of hydrolysis. In the *ex vivo* analysis, an extra peptide, TVY, was isolated. On the other hand, during *in vitro* hydrolysis the IW peptide was identified which had not been found during the *ex vivo* studies. Borawska *et al.* (2015) also analysed carp muscle tissue for antioxidant and ACE inhibitory activity by *ex vivo* digestion. ACE inhibitory activity increased with the duration of *ex vivo* digestion. After 2 hours, gastric samples inhibited ACE by 41%, and, after 15 min, the duodenal-digested samples inhibited ACE by 51% (30% after chewing). The highest activity (IC_{50} = 1.90 mg/ml) was found in the hydrolysate prepared after 2-hour 'gastric' digestion and 1-hour 'duodenal' stage. DPPH scavenging activity was higher after the 'gastric' stage (61 μM/g) than after 'duodenal' stages (40–25 μM/g). The highest ABTS scavenging activity was found after a 1-hour 'duodenal' stage (268 μM/g). Grass carp meat was analysed by Li *et al.* (2012) for antioxidant properties by enzymatic hydrolysis with alcalase and papain and, subsequently, the obtained hydrolysates were subjected to simulated digestion. Papain hydrolysates (HP) showed higher DPPH scavenging activity than alcalase hydrolysates (HA), 77.63% vs. 49.53%, respectively, with 10% DH. ABTS scavenging activity for HA and HP showed higher values than DPPH (80% on average). The highest reducing power (A_{700} = 0.613) was observed for HP with 10% DH at a concentration of 7 mg protein/ml. The Fe^{2+} chelating activity of HA and HP increased with increasing DH; moreover, the values for HA were higher than for HP, 90.53 vs. 86.51%, respectively, with 20% DH. Both DPPH scavenging activity and reducing power in HP (10% DH) decreased during *in vitro* digestion from 78.01% to 19.53% and from about 0.550 to 0.252, respectively. The reverse trend was observed for the metal-chelating activity of HP (10%), which increased to 95.73% after *in vitro* digestion. Also, grass carp meat was analysed by Chen *et al.* (2012) for antihypertensive properties. The authors separated grass carp protein hydrolysates by ultrafiltration into three fractions with MW distributions: below 3 kDa, between 3 and 10 kDa and above 10 kDa. The fraction corresponding to MW < 3 kDa had the highest ACEI activity (IC_{50} = 0.308 mg/ml), while the > 10 kDa fraction showed the lowest activity. The MW < 3 kDa fraction was further purified yielding fractions 1–6, the last of which contained the amino acid sequence of VAP. The researchers confirmed the stability of VAP under the simulated digestion conditions as well as the absence of its degradation by ACE. Carp roe protein hydrolysates (CRPHs) were analysed by Chalamaiah *et al.* (2015) using enzymatic hydrolysis with alcalase, pepsin and trypsin. The obtained hydrolysates exhibited excellent antioxidant properties. The pepsin hydrolysates showed the highest IC_{50} values (2.255 mg/ml) for DPPH radicals. In the case of ABTS radicals, the scavenging activities of the hydrolysates were the following in ascending order: trypsin (IC_{50} = 0.186 mg/ml) > pepsin (IC_{50} = 0.235 mg/ml) > alcalase (IC_{50} = 0.301 mg/ml). The reducing power and metal-chelating activity of CRPHs mainly depend on the type of protease. The metal-chelating activities of CRPHs were the following in ascending order: pepsin (IC_{50} = 0.615) > alcalase (IC_{50} = 0.948) > trypsin (IC_{50} = 1.341).

Salmon

Salmon (*Salmo salar*) is the most consumed fish in the world (FAO 2016). This fish is characterised by its excellent nutritional value, which makes it a natural product used in the prevention and treatment of NCDs (Darewicz *et al.* 2014). In *in silico*, *ex vivo* and *in vitro* analyses, Darewicz *et al.* (2014) determined the ACE inhibitory activity of the salmon protein fraction. *In vitro* studies showed that the TVY, VFPS, VTVNPYKLWLP, YALPHA, and ALPHA sequences did show ACE inhibitory activity. *Ex vivo* and *in vitro* hydrolysis of SP showed higher IC_{50} values (2.16 and 1.04 mg/ml, respectively) than MP hydrolysates (1.06 and 0.91 mg/ml, respectively). A

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wider range of ACE inhibitory capacity was obtained by *in vitro* hydrolysis. Rasyad *et al.* (2016) analysed the presence of ACE inhibitors in protamine derived from salmon by enzymatic hydrolysis with chymotrypsin, thermolysin, trypsin and pepsin. The highest inhibition was observed for trypsin hydrolysate (94.82%). Three peptide sequences were found in trypsin hydrolysate: SSSRPIR, SSRPIR, and PRRASR. All hydrolysates were rich in arginine, which makes them useful in hypertension therapy. FPH from defatted salmon backbones was obtained from enzymatic hydrolysis using a combination of eight commercially available enzymes (Corolase1 PP, Corolase1 7089, Protamex1, Papain FG, and Bromelain 400 GDU/g, Trypsin, Protex 6L, and Seabzyme L 200) and analysed for antioxidant and antihypertensive properties by Slizyte *et al.* (2016). All hydrolysates (except for trypsin hydrolysates) showed a higher ability to scavenging DPPH after 120 min hydrolysis than after 20 minutes. The highest DPPH scavenging ability was shown in FPH obtained using Protamex (38%). FPH produced with a mixture of Bromelain and Papain (BrP) after 20 min of hydrolysis exhibited the best iron-chelating ability (80%). Trypsin was most effective in producing ACE-inhibiting peptides after 120 min hydrolysis. Salmon skin collagen (SSCP) obtained by hydrolysis with Alcalase and papain was analysed by Gu *et al.* (2011). SSCP showed an IC_{50} against ACE of 1.165 mg/ml. Among the isolated peptides, the highest ACE inhibitory activity was found for the Ala-Arg and Val-Arg dipeptides: IC_{50} values of 0.06 and 0.332 mg/ml, respectively. A study of the ACE and DPP-IV inhibitory activity and oxygen radical absorbance capacity (ORAC) in salmon trimming protein (STP) hydrolysates was carried out by Neves *et al.* (2017). STP was hydrolysed with Alcalase 2.4 L and Alcalase 2.4 L in combination with Flavourzyme 500 L, Corolase PP, and Promod 144 MG. The IC_{50} value for ACE inhibitory activity ranged from 1.69 (hydrolysed with Promod 144 MG for 1 h) to 0.74 mg/ml (hydrolysed with Alcalase 2.4 L for 4 h). The ORAC values of Promod hydrolysates ranged from 587.41 to 882.58 μM TE/g for 2-h and 4-h hydrolysis, respectively. STP-C1 fractions (salmon trimming protein after 1 h of incubation with Corolase PP during simulated gastrointestinal digestion) showed an increased ACE inhibitory activity and superior antioxidant properties in comparison with the hydrolysate before fractionation. Fractions F38 and F39 showed the highest ACE inhibitory activity with IC_{50} values ranging from 0.4 mg/ml to 0.49 mg/ml, respectively. The best antioxidant properties were found for fractions F13, F14, and F25 to F27, all with ORAC values > 2000 μM TE/g. Peptides in fractions F25 and F37 were identified using the UPLC-MS/MS method. Phe-Phe dipeptides showed the highest ACE inhibitory activity with an IC_{50} value of 59.15 μM and ORAC value of 8.47 μmol TE/μmol of amino acid. *Ex vivo* and *in vitro* hydrolysis on salmon MP and SP was conducted by Borawska *et al.* (2016b). Salmon digests showed strong antioxidant activity. Among the hydrolysates, the gastric digest of SP exhibited the highest ability to scavenge DPPH radicals (8.88%), the highest ABTS scavenging activity (72.7%) as well as the most pronounced ferric ion-reducing activity (> 80%).

Trout

Rainbow trout (*Oncorhynchus mykiss*) is one of the most popular freshwater fish. Trout is considered an excellent source of calcium, omega-3 acids, essential amino acids and vitamins (Kim & Byun 2012). Protein accounts for about 18% of trout muscle tissue, which contains all exogenous amino acids (Łuczyńska *et al.* 2011). Despite the high popularity of trout and its excellent nutritional value, there are few studies on its BAP content. Rainbow trout muscle hydrolysate was analysed by Kim and Byun (2012) for ACE inhibitory activity. The peptic hydrolysate exhibited the highest ACE inhibitory activity (pepsin 0.61 mg/ml, trypsin 1.09 mg/ml, α-chymotrypsin 1.51 mg/ml). Fraction A with the amino acid sequence Lys-Val-Asn-Gly-Pro-Ala-Met-Ser-Pro-Asn-Ala-Asn was isolated from pepsin hydrolysates and showed the most potent ACE inhibitory activity with an IC_{50} value of 0.19 mg/ml. Darewicz *et al.* (2015b) determined the potential biological activity of selected proteins of rainbow trout using an *in silico* method. The *in silico* analysis covered seven rainbow trout proteins, for which simulated enzymatic hydrolysis was carried out. Rainbow trout collagen was the best source of BAPs (1999), and the greatest number of peptides with ACE inhibitory activity (992) was isolated from it. Pepsin, ficain and papain turned out to be the most efficient enzymes for digesting rainbow trout proteins into active peptides.

Tilapia

Biologically active peptides derived from tilapia (*Tilapia mariae*) proteins have been well investigated.

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However, due to their peroxidant content, hydrolysates from tilapia are highly biologically unstable (Huang *et al.* 2015). Using proteomic techniques, Huang *et al.* (2015) obtained seven proteins from tilapia frame and skin. The trypsin peptides showed multiple biological functions. For the *in silico* analysis, two proteins were selected: α-actin and collagen α-2. In α-actin, 146 peptides were detected with ACE inhibitory activity and 16 antioxidant peptides were also found. Collagen was dominated by dipeptides, mainly glyprolines with antihypertensive, antimicrobial and antiamnesic properties. The ACE inhibitors in tilapia protein were identified by Toopcham *et al.* (2015) using microbial hydrolysis with proteinase *V. holodenitrificans* SK-1-3-7. The ACE inhibitors weighing less than 5 kDa and consisting of sequences containing six to 13 amino acids showed the strongest activity. This confirms the thesis that peptides with shorter sequences of amino acids are better ACE inhibitors (Darewicz *et al.* 2014; Power *et al.* 2014). In this study the authors isolated a new peptide, MILLER, which exhibited the most potent ACE inhibitory activity with an IC_{50} of 0.12 μM. Tilapia collagen peptide (TCP) prepared by alcalase hydrolysis of tilapia skin was analysed by Zhang *et al.* (2016a) for antioxidant and hypoglycemic properties. For the *in vivo* studies, the authors used laboratory mice in which diabetes was induced. For 25 days, the laboratory mice were fed a diet augmented with TCP or metformin. The addition of TCP to the diet in a dose of 1.7 g/kg body weight reduced the level of glucose by 31.8%. In comparison, the diet containing metformin in a dose of 1 g/kg body weight reduced the glucose by 30.3%. The scientists demonstrated that the addition of TCP to the diet produces a comparable effect to synthetic antidiabetic medicines. The IC_{50} values of TCP on ABTS and DPPH scavenging activity were determined to be 1.48 and 7.97 mg/ml, respectively. A study of the antioxidant and antihypertensive properties of gelatin obtained from the tilapia skin with alcalase, bromelin, neutrase, flavourzyme, papain and trypsin was carried out by Choonpicharn *et al.* (2015). Low-molecular-weight fragments of gelatin (< 10 kDa) showed excellent antioxidant properties (ABTS, lipid peroxidation methods, FRAP and ferrous ion chelating). For ABTS, the highest activity was reported for flavourzyme and trypsin hydrolysates, namely 1413.61 and 1316.42 μg TE/mg protein, respectively. The same hydrolysates were found to be excellent inhibitors of linoleic acid oxidation (59.74 and 56.71%, respectively). Alcalase hydrolysates were the least effective ABTS scavengers. However, alcalase hydrolysates showed the highest reducing power in the FRAP assay (4.951 mM trolox/mg protein). Hydrolysis with bromelin made it possible to obtain the peptides with the highest ability to chelate ferrous ions (89.895%). All proteases used in hydrolysis showed excellent ACE inhibitory activity (> 89%).

Tuna

The great popularity of tuna (*Thunnus thynnus*) is related to its high nutritional value (Yang *et al.* 2011). This popularity of tuna has contributed to interest in the activity of its BAPs. Tuna frame protein hydrolysates (TFPH) were analysed by Lee *et al.* (2010) for antihypertensive activity. Pepsin hydrolysates showed the highest ACE inhibitory activity (88.2%). The fraction with a molecular weight of 1–5 kDa was chosen for the purification of ACE inhibitory peptides. A 21-amino acid peptide (PTFP) which showed antihypertensive activity was isolated from TFPH. Oral administration of PTFP (10 mg/kg of body weight) to rats with hypertension reduced systolic blood pressure (SBP) in laboratory animals by 21 mmHg at 6 h, which was similar to the effects of captopril (24 mmHg at 6 h). Han *et al.* (2015) analysed gelatin from the abdominal skin of yellowfin tuna (ASG) for antioxidant properties, ACE inhibitory activity and the ability to scavenge nitrites. ASG was hydrolysed with Alcalase, Protamex, Neutrase, and Flavourzyme. Alcalase hydrolysates harboured the most effective antioxidant properties, including metal ion chelating activity (86.8%), superoxide anion scavenging (SOD) (39.7% in 1 h hydrolysis), hydroxyl radical scavenging activity (37% in 3 h hydrolysis) and nitrite scavenging activity (44.4–60.7%). ACE inhibitory activity of the hydrolysates from ASG was strong, with an IC_{50} value of 1.46 mg/ml. Fraction A had the best ACE inhibitory activity, with the highest protein concentration and an IC_{50} value of 0.75 mg/ml. Hsu (2010) analysed the antioxidant activity of hydrolysates obtained from the dark fraction of tuna muscle by hydrolysis with orientase (OR) and protease XXIII. Two peptides with antioxidant properties were isolated: Leu-Pro-Thr-Ser-Glu-Ala-Ala-Lys-Tyr (978 Da) and Pro-Met-Asp-Tyr-Met-Val-Thr (756 Da). DPPH scavenging capacity expressed as relative antioxidative activity in days was higher for protease hydrolysates (79.6% in 7.89 days) than for

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orientase hydrolysates (85.2% in 7.13 days). Yang *et al.* (2011) analysed the antioxidant effect of protein hydrolysates derived from tuna head (THPH) by enzymatic hydrolysis with Alcalase. THPH showed a strong antioxidant effect: IC_{50} values for DPPH, SOD and hydroxyl radicals were 1.34, 1.2, and 2.84 mg/ml, respectively. The reducing power was 0.948 at 12.5 mg/ml. THPH at the highest concentrations showed the strongest activity against DPPH, SOD, and hydroxyl radicals: 82.85, 96.46, and 81.21%, respectively. THPH also demonstrated the ability to inhibit the oxidation of soybean oil stored at a temperature of 60°C. Je *et al.* (2009) analysed tuna liver for antioxidant and antihypertensive properties by enzymatic hydrolysis with Alcalase, Flavourzyme, Neutrase, and Protamex. All the obtained hydrolysates showed very similar antioxidant properties, which increased together with increasing concentrations: DPPH (> 80% at 5.0 mg/ml concentration), hydroxyl radical-scavenging activity (> 50% in 5 mg/ml concentration), hydrogen peroxide-scavenging activity (> 80% in 5 mg/ml concentration) and ferrous ion-chelating activity (> 80% in 5 mg/ml concentration). Moreover, all obtained hydrolysates showed similar ACE inhibitory activities (of around 36%).

Antioxidant and antihypertensive properties of BAPs derived from fish products

It is possible to extend the durability of fresh fish and to preserve its high physico-chemical and microbiological quality using technological processes. This, in turn, makes it possible to distribute fish products all around the world without loss of their health-promoting properties. Techniques involving a lowering of temperature (cooling and freezing), heat treatment (canning, cooking, fermentation and smoking), water reduction (drying, salting and smoking) and change in storage conditions (packing and cooling) ensure the safety and stability of fish products (FAO 2016). Fish products are perceived by consumers as tasty, instant, convenient and wholesome products (Ferraro *et al.* 2013).

A study of the antioxidant and antihypertensive activity of FPH obtained from the sarcoplasmic muscle of canned sardines (SPH) was carried out by Vieira and Ferreira (2017). Before hydrolysis, the FRAP value of the mixture was 146 μM TE/ml, and after hydrolysis the FRAP value of SPH was 291 μM TE/ml. The reducing power values were 1210 μg/ml before hydrolysis and 1312 μg/ml in SPH. ACE inhibitory activity also changed after hydrolysis; the IC_{50} value before hydrolysis was 604 μg/ml and 164 μg/ml afterwards. Fujita *et al.* (1995) analysed dried bonito muscle after thermolysin and gastrointestial protease digestion for ACE inhibitory activity (IC_{50} values were 29 and 41 μg/ml, respectively). Single oral administration of the thermolysin digest of dried bonito significantly reduced systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR), with a maximal reduction of 22 mmHg in SBP after 6 h at a dose of 500 mg/kg of the digest. No reduction in SBP levels was reported after single oral administration of the the gastrointestinal protease digest (dose 1000 mg/kg). Long-term administration (seven weeks) of the thermolysin digest SHR in a dose of 15 mg/kg contributed to a significant reduction of SBP. The research of Yokoyama *et al.* (1992) showed the same ACE inhibitory activity in dried bonito muscle after thermolysin digest (IC_{50} = 29 μg/ml). Phadke *et al.* (2014) analysed the impact of the duration of fermentation on the antioxidant and antihypertensive activity of peptides released *in vitro* from fermented Ngari fish. Both activities depended on the protein concentration in the product and the duration of fermentation. ACE inhibitory activity was highest after ten and 12 months at 5 mg/ml protein concentration (65.82 and 69.77%, respectively). These studies showed that traditional fermented fish product (Ngari) may be used in reducing hypertension and lipid oxidation. DPPH radical-scavenging activity, FRAP and lipid peroxidation inhibition activity was highest after 12 months at 5 mg/ml protein concentration (83.43, 2.11, and 72.14% respectively). The influence of the duration of fermentation on ACE activity was also analysed in fermented and salted anchovies by Kim *et al.* (2016). They confirmed that with increasing fermentation time, ACE inhibitory activity increased. After 12 months of fermentation ACE inhibitory activity was 67%, while at 15 months it had increased to 96%. Moreover, these authors showed that ACE activity depended on the concentration of salt in the product: at 25 mg salt/ml, ACE activity was 40% higher compared to that of the reaction solution without salt. Four new peptides, Pro-Lys (PK), Gly-Cys-Lys (GCK), Asn-His-Pro (NHP), and Asp-Gly-Gly-Pro (DGGP), showing ACE inhibitory activity were isolated from anchovies. The IC_{50} values for the ACE inhibitory activities of DGGP, GCK, NHP, and PK were 164, 178, 1172, and 4092 μM, respectively. Studies on the

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antioxidant properties of anchovies were carried out by Ovissipour *et al.* (2013). The hydrolysis was conducted using endogenous and commercial enzymes. DPPH-scavenging activity was the highest in bromelain, papain and Promod hydrolysates (15.6, 14.7, and 15.4 μM TE/g, respectively). The highest reducing power was observed in bromelain and Promod hydrolysates (7.6 and 7.5 μM TE/g, respectively). The highest ferrous chelation activity was found in bromelain and papain hydrolysates (52.5 and 51.1, respectively). The highest ABTS activity was observed in Alcalase hydrolysate (976 μM TE/g). The antioxidant activity of anchovy protein hydrolysates (APHs) was analysed by Zhao *et al.* (2017). The results showed that APHs subjected to 8 h of hydrolysis (APHs-8) exhibited the best antioxidant properties. The researchers obtained a similar value for the reducing power of APHs-8, as also reported by Ovissipour *et al.* (2013) (7.08 μM TE/g). The antioxidant activity of anchovy fins (HAHp) was analysed by Song *et al.* (2015). They determined DPPH radical-scavenging activity, reducing power and ability to chelate iron for five synthesised peptides (AGDDARPA, ESAGLHE, NKVKGELD, EMSAGLHE, and WRKKDPLND, namely HAHp1-2I, HAHp1-2II, HAHp1-2 III, HAHp1-2IV, and HAHp1-2V, respectively). HAHp1-2IV and HAHp1-2V exhibited the highest activity in scavenging DPPH radicals – 52.69 and 52.96%, respectively. HAHp1-2III and HAHp1-2V had the highest molecular weights and showed the highest reducing power (0.1145 and 0.076, respectively). The highest Fe^{2+} chelating activity was shown by HAHp1-2IV (17.61%). Stronger synergistic effects on DPPH radical-scavenging activity and ferric reducing power were shown, respectively, by HAHp1-2IV+V, about 60%, and HAHp1-2III+IV, about 0.15. Both values were higher than those for separate peptides. The study by Wiriyaphan *et al.* (2012) determined the antioxidant effect of FPH obtained from threadfin bream surimi, including the frame, bones, skin (FBS) and from refiner discharge (RD). FBS hydrolysates produced using enzymatic hydrolysis were characterised by higher antioxidant activity than their RD equivalents. Pepsin FBS hydrolysates showed the strongest ABTS scavenging activity, FRAP activity and β-carotene bleaching (about 0.45 mg TE/mg, 0.22 mM TE and 0.82, respectively). FBS and RD hydrolysates protected, to a similar degree, HepG2 cells against oxidative damage induced by tert-butyl hydroxide. This FRAP value is comparable to that exhibited by protein hydrolysates derived by Yongsawatdigul and Hemung (2010) from threadfin bream surimi by-products (221 μM TE/ml). Chalamaiah *et al.* (2013) studied the antioxidant activity of roe obtained from fish of the Labeo genus. Pepsin hydrolysates showed higher DPPH scavenging activity than trypsin hydrolysates (about 90 and 67% in 2.5 mg/ml concentrations, respectively). However, trypsin hydrolysates exhibited a higher inhibition of ABTS radical cations (32–91%) than pepsin hydrolysates (37–80%) at lower concentrations. Both hydrolysates showed strong, dose-dependent, reducing power at all concentrations; however, values reported for pepsin hydrolysates were not much higher. The highest Fe^{2+} chelating activity was observed at a concentration of 2.5 mg/ml (56.8% for pepsin and 58.9% for trypsin). The study by Gringer *et al.* (2016) was aimed at describing the antioxidant properties of low-molecular-weight compounds < 10 kDa (LMWC) compared to raw brines from traditional headed barrel-salted herring (TS). The sequence of HDF AAs is repeated in some LMWC and which may be responsible for their antioxidant activity. The authors demonstrated that adding spices during the production of pickled herring affects the antioxidant properties. Most of the antioxidant properties of raw brine have higher values than fractions < 10 kDa; for example, the highest radical scavenging was found for TS with spice (TSp; 0.42 and 0.34, respectively). Rajapakse *et al.* (2005) isolated the hepta-peptide sequence HFGBPFH from fermented marine blue mussels and named it MRSP. MRSP had the best ability to scavenge superoxide (98%), hydroxyl (96%), carbon-centered (91%), and DPPH radicals (72%) at a concentration of 200 μg/ml. Moreover, its Fe^{2+} chelating ability was 75%. The antihypertensive properties of a deka-peptide (EVMAGNLYPG) from fermented marine blue mussel (FBMS) was analysed by Je *et al.* (2005). The IC_{50} value of EVMAGNLYPG was 19.34 μg/ml. Oral administration of EVMAGNLYPG (10 mg/kg of body weight) to rats with hypertension reduced SBP in laboratory animals by 19 mmHg at 3 h, and the activity was maintained for 6 hours.

Stability of antihypertensive and antioxidant peptides

Most of the existing studies on BAPs are focused on their isolation, identification and the mechanism of action (Manikkama *et al.* 2016). The health benefits associated with the consumption of products rich in

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BAPs have been confirmed in many studies. On the other hand, there are few scientific reports on the stability of FPH during processing, storage, and consumption (Rao *et al.* 2016). Poor stability, low water solubility and low permeability through biological barriers limit their application in food products as well as the production of FPH on an industrial scale (Zhang *et al.* 2016b). The solubility of a protein/peptide depends on pH, temperature, the AA composition, and the sequence of the peptide. The high proportion of hydrophobic AAs in ACE inhibitory peptides may limit their solubility in water (Li-Chan 2015). Thus, due to the potentially poor bioavailability of such drugs, their use in the pharmaceutical industry is limited (Savjani *et al.* 2012). The main limitation of the use of BAPs in the functional food industry is their poor bioavailability as a result of gastrointestinal degradation (Madureira *et al.* 2010). Often, ACE inhibitory peptides isolated from food lose their hypotensive effect after their oral administration *in vivo* (Tsai *et al.* 2008). Enzymes, acids and the strong pH of gastric acid cause changes in the protein after its consumption (Bruno *et al.* 2013). To exert their physiological effects *in vivo*, bioactive peptides have to reach their target sites intact (Lafarga & Hayes 2016). One of the methods to prevent the degradation of BAPs against in the digestive tract is to modify them by masking the free carboxylic and amino groups (Rao *et al.* 2016).

The low number of studies on the stability of BAPs in fish makes it worthwhile to look at the impact of technological processes on the activity of peptides in other meat products (EFSA Scientific Report 2009; Lafarga & Hayes 2016).

The conditions during technological processes are variable, and temperature fluctuations are often extreme (Traore *et al.* 2012). Technological processing, addition of metal ions, ultraviolet, ultrasonic and microwave radiation as well as storage conditions causing fluctuations in the temperature and pH may change the charge and structure of peptide chains and result in their degradation (Lafarga & Hayes 2016). High pH values and temperature variations could destroy heat- and pH-sensitive AAs. Asp and Glu could be destroyed as a result of acid pH, while Cys, serine (Ser), and Thr could be destroyed by alkaline environments. High pH and temperature variations reduce the bioavailability of Lys because of an increase in the frequency of the Maillard reaction (Lafarga & Hayes 2016). Physical and structural properties of a protein/peptide could be changed as a result of freezing and low temperatures (Leygonie *et al.* 2012). Application of high-pressure processing (HPP) in the food industry is aimed at improving organoleptic and sensory properties as well as the physico-chemical properties of the final product (Lafarga & Hayes 2016). However, it is inevitable that the susceptibility of the protein to hydrolysis and digestion will be increased and the bioavailability lowered, as a result of irreversible conformational changes occurring in the protein (Garcia-Mora *et al.* 2015). Moreover, technological processes are often accompanied by thermal denaturation, which results in changes in the physico-chemical properties of the protein and FPH, mainly the loss of its solubility and functionality (Kristinsson & Rasco 2000). In addition, the structure of the polypeptide is changed, the hydrophobic surface expands (Yongsawatdigul & Park 2003) and aggregation takes place (Traore *et al.* 2012). Cold-water fish proteins are more susceptible to denaturation than those of fish from tropical waters (Kristinsson & Rasco 2000). The T-50 value, i.e., a given temperature at which 50% of the protein is denatured, depends on pH. It has been demonstrated that at pH 7, fish protein denaturation takes place at a temperature of 29–35°C, while at pH 5.5 it occurs at 11–27°C (Kristinsson & Rasco 2000). Adverse environmental conditions and chemical reactions (deamination, hydrolysis and oxidation) can contribute to reduction or inhibition of the peptide's activity.

Fish are an excellent source of BAPs. However, as FPH tend to oxidise, their production is problematic. This is related to the high content of unsaturated fatty acids, which are easily subjected to oxidation (Rao *et al.* 2016). High biological instability makes it impossible to use FPH in the medical and food industries. However, the desire to produce FPH on an industrial scale should mean that the biostability of such substances will be a focus of research in coming years (Zhang *et al.* 2016b).

A study that tested the stability of FPH against various environmental conditions, including pH, temperature and digestive enzymes is presented below.

This study was focused on the stability of protein hydrolysates from large yellow croaker (*Pseudosciaena crocea*) and was carried out by Zhang *et al.* (2016b). The impact of NaCl, temperature, pH, UV radiation, sugars, metal ions, and simulated digestion on the antioxidant activity of protein hydrolysates was studied. The obtained protein

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hydrolysates were resistant to sugar and low concentrations of NaCl as well as to K^+ and Ca^{2+} ions. Exposure to temperatures of below 60°C and the short exposure of protein hydrolysates to UV also resulted in no observable changes. Deterioration of protein hydrolysate quality was observed in response to 24-h exposure of hydrolysates to UV radiation and Fe^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+} ions. Strongly acidic and alkaline conditions did not significantly influence the antioxidant activity of the studied material. According to the authors, in order to maintain the maximum biological value of protein hydrolysates obtained from large yellow croakers (*Pseudosciaena crocea*), one should ensure the right conditions for their storage. Elavarasan *et al.* (2016) analysed carp protein hydrolysates after lyophilisation (FD-FPH) (–45°C/60 min) and drying in an oven (OD-FPH) (80 ± 2°C/48–60 h). The impact of the two drying technologies on maintaining the ACE inhibitory activity after *in vitro* digestion was studied. Structural changes caused by the drying processes included changes in colour induced by the Maillard reaction and degradation of peptide secondary structure. Before *in vitro* digestion, the ACE inhibitory activity of OD-FPH was comparable to that of FD-FPH. The OD-FPH sample after *in vitro* digestion maintained an ACE inhibitory activity that was close to the initial level (no significant changes). However, the ACE inhibitory activity of the digested FD-FPH sample was reduced to 37.5% of the initial activity. Balti *et al.* (2010) analysed the temperature stability of three novel ACE inhibitory peptides (Met-Ala-Trp, Val-Tyr-Ala-Pro, and Val-Ile-Ile-Phe) isolated from cuttlefish muscle protein hydrolysates. The results showed that 2-h incubation in the temperature range from 4°C to 100°C did not change the IC_{50} value of the studied peptides (16.32, 6.1, and 8.7 μM, respectively). It has also been demonstrated that FPH derived from cuttlefish muscle are resistant to degradation by enzymes of the digestive tract (relative ACE inhibitory activity range: 96.2–99.7%). Toopcham *et al.* (2015) determined the thermo- and pH-stability of the ACE inhibitory activity of a new peptide (MILLER) from tilapia. The MILLER peptide retained its ACE inhibitory activity in a temperature range from 100°C to 121°C and under a wide range of pH conditions (2–10, no significant changes). Also, *in vitro* digestion of MILLER peptides did not change their activity. Wang *et al.* (2008a) isolated a new peptide (VVYPWTQRF) from fresh oysters that showed ACE inhibitory activity (IC_{50} value of 66 μM/l). The study demonstrated excellent stability of the VVYPWTQRF peptide at different temperatures (4–100°C for 2 h), in variable pH (2–12 for 2 h) and during *in vitro* digestion. A similar methodology was used by Fu *et al.* (2015). They investigated the stability of ACE inhibitory activity from bovine collagen and its changes during technological treatment in the presence of gastrointestinal proteases. The ACE inhibitors maintained more than 90% of their activity after the application of different temperatures (20–100°C) and pH (2–10) and after *in vitro* digestion. Hwang (2010) analysed the stability of ACE inhibitory peptides derived from tuna cooking juice during different technological treatment and *in vitro* digestion. Non-significant changes were observed for the stability of these peptides kept for 2 h at different temperatures (20–100°C), pressures (50–300 MPa) and at variable pH (2–10). Non-significant changes in ACE inhibitory activity of the OA3 oligopeptide were also observed after *in vitro* digestion (decrease from 78.13% to 74.20%).

CONCLUSIONS

Many species of fish and fish products contain BAPs with antioxidant and antihypertensive properties. Because of their ease of isolation and wide access to the raw material, fish and fish products represent excellent ingredients in functional foods. They can play a role in the prevention of NCDs and have a positive effect on human health. In addition, ACE inhibitors are an excellent alternative to synthetic antihypertensive drugs. Manufacturers aim to preserve BAPs in the final product for the required period of time and a further goal is the retention of the *in vitro* activity of BAPs after *in vivo* studies. The present review shows that BAPs isolated from fish exhibit quite good stability when subjected to non-extreme physical conditions and after simulated *in vitro* digestion. Therefore, it is important to determine the optimal conditions under which proteins (and peptides) can be processed in order to maintain their bioactivity. It should be remembered that each protein reacts individually to extreme conditions in the food industry; the conditions that reduce the activity of one peptide may intensify the actions of other peptides. Future studies should be aimed at a fine-tuning of the optimal conditions under which BAPs can be processed while maintaining their bioactivity, e.g., after digestion *in vivo*.

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