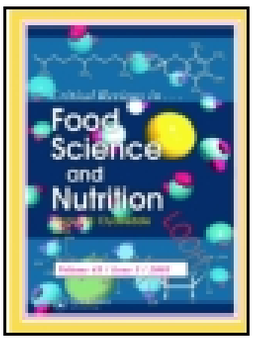

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Antihypertensive Activity of Fish Protein Hydrolysates and its Peptides

Yathisha UG, Ishani Bhat, Iddya Karunasagar, Mamatha BS*

Nitte University Centre for Science Education and Research (NUCSER), NITTE (Deemed to be University), Paneer Campus, Kotekar-Beeri Road, Deralakatte, Mangalore, 575-018, Karnataka, India.

***Corresponding Author:** Mamatha B.S., (Ph.D),
Assistant Professor,
Nitte University Centre for Science Education and Research (NUCSER),
NITTE (Deemed to be University),
Paneer Campus, Kotekar-Beeri Road,
Deralakatte, Mangalore, 575-018,
Karnataka, India.
E-mail: mamatha.bs@nitte.edu.in

Abstract:

The rising interest to utilize nutritionally exorbitant fish proteins has instigated research activities in fish waste utilization. The development of newer technologies to utilize fish waste has fostered use of bioactive value-added products for specific health benefits. Enzymatically obtained Fish Protein Hydrolysate (FPH) is a rich source of biologically active peptides possessing anti-oxidant, anticancer, antimicrobial and anti-hypertensive activity. Isolating natural remedies to combat alarming negative consequences of synthetic drugs has been the new trend in current research promoting identification of antihypertensive peptides from FPH. In this review, we aim to culminate data available to produce antihypertensive peptides from FPH, its composition and potential to be used as a therapeutic agent. These purified peptides are known to be rich in arginine, valine and leucine. Reports reveal peptides with low molecular weight (<1kDa) and shorter chain length (<20 amino acids) exhibited higher antihypertensive activity. As these peptides have proven Angiotensin Converting Enzyme - I inhibitory activity *in vitro* and *in vivo*, their potential to be used as antihypertensive drugs is outrageous. However, current focus on research in the field of molecular docking is necessary to have improved understanding of interaction of the peptides with the enzyme.

Key words

Fish; Proteolytic enzyme; Fish protein hydrolysate; Angiotensin converting enzyme; Antihypertensive peptides.

Introduction

Fish being the main protein source of food world-wide, the potential for fishing is so high that in Indian coast, fishing is a major occupation employing over 14 million people making India the second largest producer of fish. Every year over 91 million tons of fish are harvested, of which 29.5% is transformed into fishmeal. Depending on the level processing and type of fish, processing results in 20-80% wastage. Possibly more than 50% of the remaining fish tissue is processing waste and not used as food which creates burdensome disposal problems and environmental concerns. Fish wastes such as viscera, heads, frames, cut-offs, bone, skin contain nutritionally valuable macro and micro-nutrients. 7.3 million tons of fish processing waste is estimated to be discarded every year (Pierre et al., 2012). Discarding the waste without any attempt of recovering nutrients is posing to be a national wastage. Nutritionally rich fish waste can thus be exploited for the production of value added products. Since protein is the second-most abundant constituent in fish, fish waste possesses high protein content having exorbitant nutritional value (Sidhu, 2003) and can supply at least 15 - 20% of the average animal protein intake. By applying enzyme technology for protein recovery in fish processing, it may be possible to produce a broad spectrum of food ingredients or industrial products for a wide range of applications (Rustad et al., 2011).

Enzymatic hydrolysis of fish proteins has been employed as a principle method for converting fish waste into valuable products for the pharmaceutical and food industries (Vilhelmsson, 1997). Fish Protein Hydrolysate (FPH) is produced from fish and its waste using proteolytic enzymes like alcalase, protamex, flavourzyme, trypsin, α-chymotrypsin, pepsin, papain, proteinase K, neutrase and bromelain (Ahn et al., 2010; Beaulieu et al., 2009; Dong et al., 2005; Duan, et al., 2010; Pastoriza et al., 2004; Wasswa et al., 2007). Proteolytic enzymes

break protein into small fragments of peptides containing 2-20 amino acids. Hydrolysates thereby embody amino acids in the most available form conveniently growing to be a good aid for various biological functions of human body (Neklyudov et al., 2000). Due to the reduced peptide size, FPH not only has improved digestibility (Kristinsson and Rasco, 2000) but also exhibits noteworthy functional properties moulding to be a promising source for nutraceutical and pharmaceutical applications (Oliva et al., 1999,).

The functional properties that FPH exhibit are affiliated to the mere existence of specific protein fragments encrypted within the sequence of the parent protein which when processed and digested get released to the active functional site. In addition to acting as sources of nitrogen and amino acids, these bio-functional peptides have potential physiological functions like antihypertensive, immunomodulatory, antibacterial and antithrombotic activity (Murray and FitzGerald, 2007). These revelations in the recent years has grabbed even more attention by researchers urging to develop techniques to produce bioactive peptides with specific health benefits. Recent focus has been on production of Angiotensin Converting Enzyme (ACE) inhibitory peptides exhibiting antihypertensive activity from fish and its waste.

Every year approximately 17.7 million people die of CVD (WHO 2014). Hypertension refers to persistent raised blood pressure in the arteries and is a long term medical condition initiating major risk factors for Cardio-Vascular Diseases (CVD). Renin-Angiotensin System (RAS) plays a key role in maintaining the blood pressure and homeostasis by performing salt balance (Griendling et al., 1993). RAS blood pressure regulation involves two specific reactions catalyzed by Angiotensin Converting Enzyme (ACE). In the first reaction ACE converts the Angiotensin-I, a decapeptide to Angiotensin-II, an octapeptide which plays a significant role in regulating blood pressure by performing vasoconstriction and retaining salt. In the second

reaction ACE converts bradykinin, a vasodilator into inactive fragments (Goodfriend et al., 1996). This leads to a raise in blood pressure and these metabolic pathways show that activity of ACE is responsible for hypertension. Henceforth, inactivating ACE is considered as the first line of therapy to treat hypertension (Welderufael et al., 2012 and Patchett et al., 1980). The ability for some molecules to inhibit ACE is useful in therapeutic approach of treating hypertension. Many synthetic antihypertensive drugs such as captopril, enalapril etc. are commercially available (Chen et al., 2013). But these antihypertensive drugs were also proven to cause many side effects like cough, taste disturbance, renal problem and angioneuricoedema (MacGreror and Antonios, 1995). Due to the adverse health effects of the synthetic antihypertensive drugs, the search for safe natural antihypertensive agents such as those derived from marine species is gaining importance. This review mainly focusses on compiling the recent researchers and developed techniques to produce ACE inhibitory peptides from fish proteins and discusses the production, composition and activity of the same.

1. Production of FPH by enzymatic hydrolysis

Hydrolysis of fish proteins can be achieved using acids, alkali, enzymes or heat (Himonides et al., 2011). However, use of acid or alkaline hydrolysis and thermal hydrolysis results in loss of nutrients, damage to amino acid profile and reduction in consumer acceptability. Thus, enzymatic hydrolysis is the most accepted and suitable method of producing FPH for several reasons. Use of food grade enzymes not only increases the consumer acceptability but also succors to obtain specific peptides by performing site specific cleavage. In addition, the manufacturing process becomes much more controllable. Selection of appropriate proteolytic enzyme is an important factor for the release of potent antihypertensive peptide from fish

protein. Commercial enzymes proteolytic in nature are generally used which are isolated from microbial, plant, and animal sources (Simpson et al., 1998; Toopcham et al., 2015; Perez-Galvez et al., 2016; Salampessy et al., 2017). These include alcalase, flavourzyme, pronase, neutrase, protamex, bromelain, cryotin F, protease N, protease A papain, α-chymotrypsin, pepsin, trypsin acid proteases and neutral proteases (Je et al., 2009; Dong et al., 2005; Beaulieu et al., 2009; Duan et al., 2010; and Wasswa, et al., 2007; Gu et al., 2011; Hoon et al., 2008; Raghavan and Kristinsson, 2009; Ping et al., 2014; Lan et al., 2015). Apart from the above-mentioned enzymes, use of esperase enzyme for the production of squid FPH was noted Mosquera et al., (2015). Ko et al., (2016) reported the use of kojizyme for flounder FPH production. Visceral extracts of a few fishes like catfish, cuttlefish, smooth hound fish, grey trigger fish, golden mullet fish and goby fish have been used with an intention of improving the functional properties of the FPH (Ketnawa et al., 2017; Balti et al., 2015; Nasri et al., 2013). Reports with the usage of porcine and human gastrointestinal enzymes for hydrolysis exist, however, with very little clarity on functional properties of the FPH (Browska et al., 2015; Darewiez et al, 2014).

Enzymes are known to have required performing conditions among which pH and temperature are the most important. Every enzyme requires a specific optimum pH at which it will perform the best. For instance, acid proteases work at pH 2.7 whereas neutral proteases work at pH 7.0 (Douglas and Moodie, 2016). However, the optimum temperature they need is not largely varying; 45 ^{o}C and 48 ^{o}C respectively. The optimum pH and temperature requirements of commercially available enzymes to produce FPH is listed in Table 1. Pepsin requires the lowest pH (2.0) and lowest temperature (37 ℃) of all. On the contrary, trypsin and chymotrypsin require the highest pH (8.0) and highest temperature (60 ℃) of all. Use of enzymes, optimum conditions maintained, time and degree of hydrolysis greatly affect the amino acid composition of the FPH.

Amino acid profile of fish proteins consumed, has significant role in various biological and physiological activities of human body and in maintaining health. As described in Table 2, researchers have discovered the amino acid composition of FPH obtained from different fish species like grass carp (Zhang et al., 2009), atlantic salmon (Gu et al., 2011), cat fish (Yin et al., 2010), sardinelle (Bougatef et al., 2008), tuna (Hoon et al., 2008), shark meat (Wu et al., 2008), tilapa (Raghavan and Kristinsson, 2009), alaska pollack (Nakajima et al., 2009), ribbon (Ping et al., 2014), seela (Nazeer and Deeptha, 2013), jelly (Xin et al., 2012), lizard (Lan et al., 2015), chum salmon (Jung et al., 2014), goby (Nasri et al., 2013), and tuna (Nilang et al., 2005). Despite fish proteins classified as complete proteins (proteins containing all the amino acids), the amino acid profile of FPH mainly depends on factors such as fish species, part of fish, enzyme used for hydrolysis and duration of hydrolysis (Klompong et al., 2009). The essential amino acids have been found abundantly in jelly FPH and cat FPH (Sathivel et al., 2003; Sathivel et al., 2005 and Yin et al., 2010).

From Table 2 it is observed that amino acid composition varies in accordance with the fish species. Although, chum salmon is appearing to have higher contents of glutamine other fishes like carp, jelly, alaska pollack and others did not show any traces of it. Jelly, tuna and cat FPH showed higher amounts of glycine whereas chum salmon showed no traces of it. Similarly, higher amounts of leucine in alaska pollack (Nakajima et al., 2009), histidine in sardinelle (Bougatef et a., 2008), arginine in seela and tyrosine in ribbon FPH was noted (Nazeer and Deeptha, 2013). However, most of the fish species show superlative amounts of arginine and valine throughout. Conversely, tryptophan, cystine and hydroxyproline was reported in very few fish species. Hydroxyproline was reported only in chum salmon and cat FPH (Jung et al., 2014; Yen et al., 2010). The variation of amino acid composition in different species can be clearly

understood in 2 types of salmon; chum salmon and atlantic salmon. Chum salmon FPH shows highest amount (36%) of glutamine and atlantic salmon FPH (15%) shows highest amount of glycine. However, chum salmon shows no traces of glycine and atlantic salmon shows no traces of glutamine (Jung et al., 2014; Gu et al., 2011). From Table 2 it was observed that glutamine, hydroxyl b proline and tryptophan were not detected in any fish species or its parts except chum salmon (Jung et al., 2014). Therefore, amino acid composition in fish varies from species to species.

As discussed earlier selection of enzymes has a key role in amino acid extraction. From Table 2 it is observed that, although FPH made using pepsin and protamex had greater amounts of glycine (Zhang et al., 2009; Nakajima et al., 2009; Yen et al., 2010), amino acid profile of ribbon FPH obtained using pepsin showed lower amounts of glycine and a notable increase in amounts of tyrosine was observed (Nazeer and Deeptha, 2013). FPH produced using alkalase also composed of higher glycine content (Gu et al., 2011; Akagundaz et al., 2014). The site of cleavage of the specific enzymes used is the key to understanding the amino acid profiling pattern. Pepsin cleaves at aromatic and hydrophobic amino acids whereas trypsin cleaves arginine and lysine. However, a clear conclusion on the effect of enzyme solely on the amino acid profile of FPH could not be derived as amino acid profile is majorly dependent on the part of the fish used for hydrolysis. We noticed that FPH produced using alkalase enzyme was extracted from skin of atlantic salmon and sea breams (Gu et al., 2011; Akagundaz et al., 2014). Supporting the fact of fish skin to be rich in glycine, FPH extracted from the skin of cat fish also showed higher amounts of glycine (Yen et al., 2010). Contrary to this, FPH extracted from skin of chum salmon using trypsin showed no traces of glycine (Jung et al., 2014). FPH extracted from the viscera of sardinelle using moulinex constituted large amounts of histidine, arginine and

lysine (Bougatef et al., 2008). However, FPH extracted from muscle and whole fish showed higher contents of glycine (Zhang et al., 2009; Xin et al., 2012; Nakajima et al., 2009). The amino acid content henceforth depends mainly on the selection of enzyme, fish type and its parts.

2. **Isolation of ACE-I inhibitory peptides from FPH**

Similar to natural digestive processes, in the initial steps of protein hydrolysis, long protein chain is efficiently broken down to smaller units of peptides which can be separated from the non-digested proteins and oil by liquid phase processing. The FPH produced containing antihypertensive components is further processed to isolate ACE inhibitory peptides. In a study conducted on boar fish, pH shift method was used to extract proteins by precipitating fractions out at definite pH (Hayes et al., 2016). By adjusting the pH to pI of the protein fractions it is possible to have a moderate separation of the proteins from other bodily constituents of fish. Reports available have separated the hydrolysed peptides based on their molecular weights by ultrafiltration and column chromatography (Mosquera et al., 2015). In a study conducted by Park et al., (2016), used cation exchanger for the purification of peptides of size <1kDa followed by Revers Phase - High Performance Liquid Chromatography (RP-HPLC). High Performance Liquid Chromatography (HPLC) has enabled researchers to obtain 93% pure ACE inhibitory peptides. Not only purity has been obtained, but the size exclusion principle also has helped researchers separate the peptides according to its molecular size (Abdelhedi et al., 2016; Zhang et al., 2017). Henceforth, using these purification steps, lower molecular weight peptides can be isolated which have been shown to exhibit higher antihypertensive property. The peptide profiling was done in many studies by the method of Liquid Chromatography - Mass Spectrophotometer (LC-MS) (Yi et al., 2017; Thuangthong et al., 2017). However, to determine

the amino acid composition of the FPH reports suggest the use of amino acid analyzer (Park et al., 2016).

Many, researchers have worked on identifying and isolating the ACE inhibitory peptides from protein rich sources such as bovine blood plasma (Hyun and Shin, 2000), prawn (Suetsuna et al., 2000), egg-yolk (Sakanaka et al., 2004), soybean fermented products (Rai et al., 2016; Nongonierma et al., 2017), fermented milk products (Rai et al., 2017), almonds (Mirzapour et al., 2017) and cauliflower (Xu et al., 2016). In the recent years, protein rich processing waste from fish is gaining consideration as valuable resource of antihypertensive agents. ACE inhibitory peptides are reported to have been extracted from marine species like krill (Park et al., 2016), pacific cod (Ngo et al., 2016), sardinelle (Jemil et al., 2017), hound (Abdelhedi et al., 2016), goby (Nasri et al., 2013), boar (Hayes et al., 2016), alaska pollack (Nakajima et al., 2009), ribbon (Ping et al., 2014), lizard (Lan et al., 2015) and different species of salmon (Gu et al., 2011; Jung et al., 2014; Neves et al., 2017). Besides, several studies have reported ACE–I inhibitory peptides derived from other salt water fishes such as tuna (Hoon et al., 2008; Je et al., 2009; Martínez-Alvarez et al., 2016) and shark (Wu et al., 2008). Not only marine and salt water fishes, but also fresh water fishes like grass carp (Zhang et al., 2009; Yi et al., 2017), carp (Zhang et al., 2017), pipe (Wijesekara et al., 2011) and distinct species of tilapia (Raghavan and Kristinsson, 2009; Roslan et al., 2017; Thuanthong et al., 2017) also have been used to produce ACE inhibitory peptides. Fractionated peptides of both fresh and salt water fishes showed higher antihypertensive activity (Hoon et al., 2008; 2006; Ping et al., 2014) compared to crude FPH (Theodore and Kristinsson 2007; Bougatef et al., 2008).

3. Antihypertensive activity of ACE-I inhibitory peptides

Antihypertensive activity of the FPH and isolated peptides derived from a variety of fishes is shown in Table 3. It is observed from the table that chain length, molecular weight and molecular interaction of the peptides have a key role in exhibiting antihypertensive activity. The antihypertensive potency of fish-derived peptides is expressed as IC_{50} value which indicates the half maximal inhibitory concentration of peptides that can inhibit 50% of ACE activity.

In vitro studies have shown that crude FPH extracted from different fish species showed ACE-I inhibitory activity (Theodore and Kristinsson 2007; Ali et al., 2008; Je et al., 2009; Raghavan and Kristinsson, 2009; Abdelhedi et al., 2016; Martinez-Alvarez et al., 2016; Perez-Galvez et al., 2016; Thuanthong et al., 2017). FPHs produced by Martinez-Alvarez et al., (2016) from sardine and tuna industrial by-products have shown remarkable ACE inhibitory activity (IC_{50} values of 1.16mg/ml and 0.24-0.27mg/ml respectively).

Researchers have been reporting that peptides isolated from enzymatically hydrolyzed crude FPH have greater ACE-I inhibitory activity (Hoon et al., 2008; Wijesekara et al., 2011; Nasri et al., 2013; Lan et al., 2015; Zhang et al., 2017). Gu et al., (2011), studied the comparison of isolated ACE inhibitory peptides (Ala-Pro and Val-Arg) and crude FPH from salmon skin collagen. It was found that ACE inhibitory activity of Ala-Pro (IC_{50} = 0.060 mg/ml) and Val-Arg (IC_{50} = 0.332 mg/ml) showed 20 and 4-folds higher activity than that of crude salmon skin collagen protein (IC_{50} = 1.165 mg/ml).

ACE inhibitory peptide (Gly-Glu-Pro-Asp-Ala) was identified by Xin et al., (2012) extracted from the jelly whole FPH. The 2kDa peptide showed IC_{50} value of 1.28 mg/ml in *in vitro* conditions. Similar chain length peptide (Gly-Met-Lys-Cys-Ala-Phe) extracted from lizard whole FPH also showed IC_{50} value at 45.7µM indicative of antihypertensive activity (Lan et al., 2015). Other peptides i) His-Leu-Ala-Leu-Thr (553Da), ii) Arg-Glu-Leu-Ala-Gly-Pro (640Da) and iii) Glu-Leu-Ser-Ala-Pro (515Da) extracted from horse mackerel whole FPH showed IC_{50} values of 5.11µM, 6.24µM and 7.08µM (Garcia-Moreno et al., 2015). In the same study peptides i) Glu-Leu-Val-Gly-Val (515Da), ii) Leu-Val-Ala-Pro-Ala-Asn (583Da), iii) Tyr-Leu-Gly-Trp (537Da) and iv) Val-Ala-Met-Pro-Phe (563Da) isolated from small-spotted catshark whole FPH showed IC_{50} value of 0.5, 0.9, 0.09 and 0.44µM respectively. Similar study by Salampessy et al., (2017), yielded varying chain length peptides (3-7 amino acids) of molecular weight 374-858Da showing IC_{50} value of 0.01-0.24mg/ml. Shorter chain length peptides i) Ala-Arg (245Da), ii) Ala-Val (188Da) and iii) Ala-Pro-Glu-Arg (471Da) were isolated from trevally whole FPH showed IC_{50} value of 448, 371 and 190µM respectively (Salampessy et al., 2015). A study conducted by Nasri et al., (2013), also suggested ACE inhibitory activity (IC_{50} value of 1.36mg/ml) of shorter chain length peptides among the peptides isolated from goby whole FPH using A21proteases. The fractionated peptides were identified as i) Ala-Arg-Ser ii) Val-Val-Ala-Pro-Phe-Ala-His-Gly-Thr iii) Arg-Ser-Thr-Ala iv) Phe-Tyr-Pro-Pro and v) Arg-Lys-Ser-Ala-Gly. The author suspected higher ACE inhibitory activity of shorter fractions due to their resistive power against simulated gastro intestinal digestion.

Attempts to extract antihypertensive peptides from individual parts of fishes like skin, muscle, viscera and such others were also reported. ACE inhibitory activity was reported in crude FPH extracted from skins of jumbo squid tunics (MW<1kDa, IC_{50} value 0.096mg/ml),

basa fish (IC_{50} value of 1.417mg/ml), nile tilapia (MW-1.1kDa, IC_{50} value of 1.2mg/ml) and giant cat fish (Mosquera et al., 2015; Thuangthong et al., 2017). Researchers have also reported ACE inhibitory activity of isolated peptides from FPH. A long peptide Gly-Arg-Gly-Ser-Val-Pro-Ala-Hyp-Gly-Pro (<1kDa) extracted from squid skin FPH showed IC_{50} value of 478µM (Aleman et al., 2013). Jung et al., (2014), isolated a 770Da peptide, Gly-Leu-Pro-Leu-Asn-Leu-Pro from skin of chum salmon FPH which showed IC_{50} value of 18.7µM. Another study in salmon skin FPH yielded peptides of varying length (free amino acids, dipeptides and peptides of 4-8 amino acids). These peptides have shown a range of ACE inhibitory activity from IC_{50} 0.098-1.191mM of which, the free amino acids (Arg and Tyr) showed highest activities (IC_{50} values of 0.098mM and 0.132mM respectively). Two peptides isolated from skin of mackerel i) Phe-Gly-Asn and ii) His-Gly-Pro-Leu of molecular weights 337 and 423Da respectively showed significant ACE inhibitory activity (Khiari et al., 2014). Two other peptides i) Gly-Ala-Ser-Ser-Gly-Met-Pro-Gly (662Da) and ii) Leu-Ala-Tyr-Ala (436Da) isolated from skin of pacific cod FPH showed IC_{50} values of 6.9µM and 14.5µM respectively (Ngo et al., 2016). The author reported better ACE inhibitory activity of the peptides to be due to better protein-ligand interaction revealed from docking study.

A study on FPH extracted from goby fish muscle was conducted by Nasri et al., (2013). They compared the ACE inhibitory activity of FPH obtained by using visceral enzyme extracts from different fishes (smooth hound, grey trigger, golden mullet and goby fish) with FPH obtained by using bovine trypsin. The ACE inhibitory activity shown by FPH obtained by using golden mullet visceral extract (IC_{50} 0.13mg/ml) was higher than FPH obtained by using other extracts (IC_{50} 0.7-0.9mg/ml) and bovine trypsin (IC_{50} 1.05mg/ml). Three dipeptides, Cys-Phe, Glu-Tyr and Phe-Glu isolated from shark muscle were found to have ACE inhibitory activity

with IC_{50} values of 1.96, 2.68 and 1.45µM respectively (Wu et al. 2008). Similar smaller peptides were isolated from muscle of salmon FPH, i) Ile-Trp, ii) Ile-Tyr, iii) Thr-Val-Tyr, iv) Val-Trp, v) Val-Pro-Trp and vi) Val-Tyr with IC_{50} values ranging from 0.19-1.04mg/ml (Darewiez et al., 2014). A tetra peptide, Arg-Tyr-Arg-Pro showing IC_{50} value of 52µM was also isolated from muscle of lizard FPH (Sun et al., 2017). Toopcham et al., (2015), reported a longer peptide, Met-Ile-Leu-Leu-Leu-Phe-Arg (905Da) isolated from tilapia muscle also exhibiting ACE inhibitory activity (IC_{50} value 0.12mg/ml). Similar peptides (4-6 amino acids) were isolated from muscle of snakehead FPH (1.3-2.8µM) and thornback ray FPH (12.50-27.07µM) (Ghassem et al., 2014; Lassoued et al., 2015; Lassoued et al., 2016). Two longer peptides i) Tyr-Asn-Leu-Lys-Glu-Arg-Tyr-Ala-Ala-Tyr and ii) Tyr-Asn-Arg-Leu-Pro-Glu-Leu isolated from muscle of carp FPH also showed IC_{50} values of 1.35µM and 3.42µM respectively (Zhang et al., 2017). Among the two peptides isolated from the muscle of pipe FPH, peptide Thr-Phe-Pro-His-Gly-Pro was found to exhibit better ACE inhibitory activity (IC_{50} value 0.6mg/ml) than peptide His-Trp-Thr-Thr-Gln-Arg (IC_{50} value 1.44mg/ml) (Wijesekara et al. 2011). FPH (339-7000Da) extracted from carp fish muscle exhibited similar ACE inhibitory activity with IC_{50} values ranging from 1.15-1.53mg/ml (Elavarasan et al., 2016). Although, reports follow the observation of higher antihypertensive activity exhibited by shorter chain length peptides, longer chain length peptides also show significant ACE inhibitory activity contradictory to the observation. However, molecular weights of the peptides play a vital role in inhibiting ACE.

ACE inhibitory activity from inedible parts of the fish such as visceral parts of hound fish (IC_{50} value of 75µg/ml) and sardinelle (IC_{50} value 0.8-1.2mg/ml), backbones of ribbon fish (IC_{50} value 5.6µM) and yellow fin sole (IC_{50} value 28.7µg/ml), heads of salmon, flathead, silver warehouse and barramundi have been reported (Abdelhedi et al., 2016; Bougatef et al., 2008;

Ping et al., 2014; Nurdiani et al., 2016). Industrial effluents of cuttlefish were treated with alcalase and ACE inhibitory peptides (7 amino acids) were isolated that showed IC_{50} value of 1.92mg/ml (Amado et al., 2014). Fractions from salmon frame hydrolysate were able to exhibit not only ACE inhibitory activity but also reduced renin activities (Girgih et al., 2016). Production of ACE inhibitory peptides from fishes can thus be used as a method to manage wastes obtained from fish processing industries.

The above reports suggest that low molecular weight peptides (<1kDa) exhibit better ACE inhibitory activity (Wijesekara et al., 2011; Aleman et al., 2013; Mosquera et al., 2015; Garcia-Moreno et al., 2015; Hayes et al., 2016; Salampessy et al., 2017). However, peptides of 3-5kDa showed noticeable ACE inhibitory activity (Akagündaz et al., 2014; Sun et al., 2017). Salampessy et al., (2015), suggested that, peptides of molecular weight <5kDa have higher inhibitory potential. It was observed that shorter chain length peptides (2-4 amino acids) had higher inhibitory potency than longer chain length peptides (5-12 amino acids) (Wu et al., 2008; Ping et al., 2014; Salampessy et al., 2015; Ngo et al., 2016). Since shorter peptides are absorbed directly in the gut, they escape the degrading effects of gastro intestinal enzymes and henceforth are available at the site of action. To understand the permeability of the ACE inhibitory peptides in the gut, *in vitro* studies with simulated gastrointestinal digestion and absorption in gut cell lines was reported in one article. Four tripeptides were isolated from muscle of tilapia FPH and permeability assays were performed in Caco2 cells. The permeate was reported to show IC_{50} values ranging from 0.29-1.23μM (Toopcham et al., 2017). However, longer peptides also showed impressive antihypertensive activity (Hoon et al., 2008). In addition, Neves et al., (2017), reported higher ACE inhibitory activities of free amino acids Tyr and Arg. The presence of Arg and Val in the peptides showing ACE inhibitory activity was noted. In a molecular

docking study, Salampessy et al., (2015), suggested stronger affinity of inhibitory peptides with Arg and Val as C-terminal residues towards ACE. The need for understanding the bioavailability of these ACE inhibitory peptides has provoked the researchers to study the behavior and effects of the peptides in *in vivo* systems.

A few *in vivo* studies have been reported on the ACE inhibitory activity of peptides from FPH. A study on hypertensive rats fed with FPH from cod, haddock, saithe, whiting, plaice and halibut reported antihypertensive effects at low dosage (Jensen et al., 2014). *In vivo* experimental results indicate that the ribbonfish backbone FPH peptides extracted using acid protease could be used for the development of antihypertensive agent (Ping et al., 2014). Out of the four different peptide fractions that were collected by ultra-filtration (1. MW <1 kDa; 2. MW = 1-5 kDa; 3. MW=5-10 and 4. MW > 10 kDa), fraction 1 showing higher ACE inhibitory activity was further purified and identified as, Leu-Trp which showed significant decrease in systolic blood pressure from 181mmHg to 161mmHg in 4 hours in spontaneously hypertensive rats. Similar studies on hypertensive rats fed with FPH, ACE inhibitory peptides fractionates, and free amino acids obtained from salmon skin was able to lower heart rate along with systolic and diastolic blood pressure (Neves et al., 2017). Baiti et al., (2015), demonstrated the antihypertensive activity of an ACE inhibitory peptide, Val-Glu-Leu-Tyr-Pro in hypertensive rats. In a single dose administration (10mg/kg) the peptide was able to exhibit suppressive effect on blood pressure even at 8th hour post administration. However, the highest decrement in the systolic (27.6mmHg) and diastolic (14.7mmHg) was observed at 2^{th} and 4^{nd} hour respectively. *In vivo* study was reported on antihypertensive peptides from grass carp. Peptides from grass carp FPH was able to reduce 27mmHg (low dose) and 43mmHg (high dose) systolic blood pressure with 50mg and 100mg/kg body weight respectively (Chen et al., 2016). With a dose of 200mg/kg

body weight, fractions from salmon frame were able to lower 21mmHg systolic blood pressure in 2h of administration in hypertensive rats (Girgih et al., 2015). In another study where, hypertensive rats were orally administered with ACE inhibitory peptides from chum salmon, highest decrement in blood pressure was observed between 3-6hr post administration (Jung et al., 2014). Peptides from skate skin were able to exhibit sustained antihypertensive effects post 20 days' oral administration (Ngo et al., 2016). Another long-term study on hypertensive rats reported antihypertensive activity (reduced renin production in the kidneys) of Ala-His-Leu-Leu from loach FPH, without any allergic reaction for 2months (Li et al., 2016). In addition, a study on diabetic rats also reported antihypertensive activity of peptides derived from zebra blenny (Jemil et al., 2016).

In vivo studies reveal the bioavailability of reported ACE inhibitory peptides. From the reports, it has been observed that most of the peptides exhibiting antihypertensive effects in *in vivo* systems are short chained and low in molecular weight supporting the theory of easier absorption of shorter peptides. However, *in vitro* studies have successfully reported ACE inhibitory activity of longer peptides too. Henceforth, it is required to access the bioavailability of longer ACE inhibitory peptides in *in vivo* systems. The interaction of the peptide with the enzyme at the active site must be completely understood to predict the potential of the inhibitory peptide designed. Easier approach to attend such a case would be to study the molecular docking.

Conclusion

FPH is an efficient way of utilizing fish waste and it is not surprising to discuss the health benefits posed by fish proteins. Enzymatic hydrolysis of fish proteins yields ACE inhibitory peptides exhibiting antihypertensive activity tested *in vitro* and *in vivo*. In this review, optimum conditions for hydrolysis, amino acid composition and antihypertensive activities of FPH and its peptides are reported. Amino acid composition of protein hydrolysates depends on the fish species, part of the fish and proteolytic enzymes used. Smaller peptides with lower weight obtained by fractionations of enzymatically hydrolyzed fish protein showed higher antihypertensive activity. Since, presence of arginine and valine in the C-terminal of the peptides increases their ACE inhibitory efficiency, use of enzymes that specifically cleave arginine like trypsin can probably help fabrication of better ACE inhibitory peptides. However, the area of molecular docking studies needs focus as it can reveal the actual interaction of the peptides and ACE. Fish derived bio-active peptides have potential as nutraceuticals and pharmaceuticals due to their effectiveness in prevention and treatment of hypertension. Further exploration on potent natural antihypertensive peptides from FPH needs *in vivo* and clinical confirmation to make these peptides as a commercially available drug.

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List of Figures:

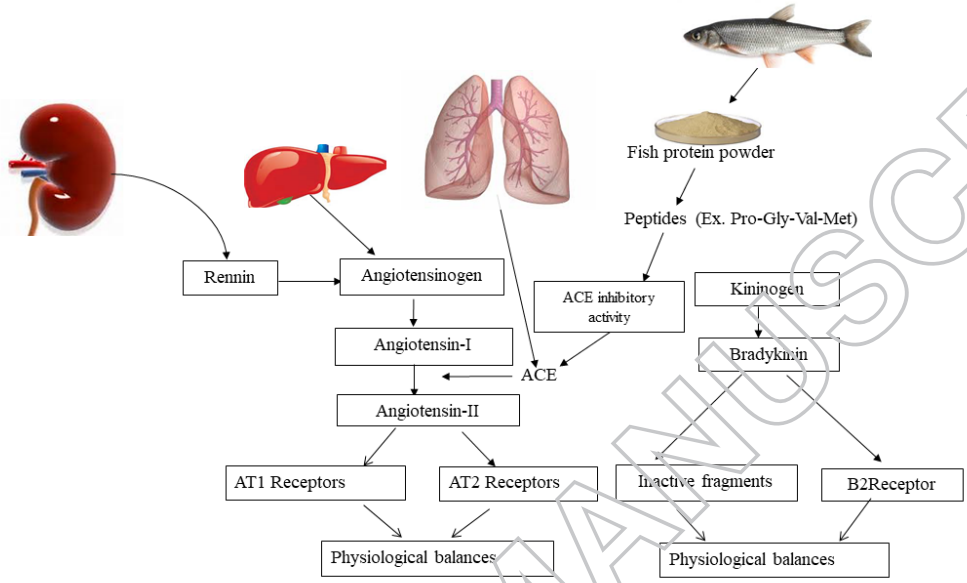

Figure 1. Mechanism of Renin Angiotensin System and role Angiotensin-I converting enzyme activity

Table 1. Optimum condition for the preparation FPH using different enzymes

Enzymes	Buffer solution	pH	Temperature (^{o}C)	References
Alcalase	0.1M PB	8.5	50	Wijesekara et al., 2011
Flavourzyme	0.1M PB	7.0	50	Raghavan and Kristinsson, 2009
Papain	0.1M PB	6.5	50	Jae et al., 2006
α-chymotrypsin,	0.1M PB	8.0	60	Jung, et al., 2006
Pepsin,	0.1MGH	2.0	37	Aleman et al.,2013
Trypsin	0.1M PB	8.0	60	Jung et al., 2014
Acid proteases	0.1M PB	2.7	45	Ping et al., 2014
Neutral proteases	0.1M PB	7.0	48	Lan et al., 2015
Protamex	0.1M PB	7.5	22	Theodore and Kristinsson 2007
Cryotin-F	0.1M PB	8.0	45	Raghavan and Kristinsson 2009
Esperase	0.1M PB	8.0	60	Akagundaz et al., 2014
Bromelain	0.1MGH	5.5	55	Li et al., 2016

PB**,** Phosphate buffer, GH, Glycine-HCL buffer

Table 2. Amino acid composition of various fish protein hydrolysate

Amino acids	carp fish (Zhang et al., 2009)					chum salm	jelly fish	alaska pollack (Na	atlantic salm	sardinelle (Bougatef	tuna (Nilang
	Crud	Ethanol concentration 2	4	6	8						
Aspartic acid /Asparagine	4.41	6.41	6 7.1	2.45	2.26	6.41	6 7.1	16.9	9.45	24.01	0.67
Threonine	1.10	3.19	4 1.6	0.92	1.18	3.19	4 1.6	24.2	3.68	13.58	0.74
Serine	3.81	4.49	3 0.9	1.95	2.33	4.49	3 0.9	6.2	5.52	14.25	6.67
Glutamicacid	9.61	8.09	11 4.2	4.89	2.29	8.09	11 4.2	29.0	13 .90	26.38	1.52
Glutamine	-	36.12	-	-	-	36 12	-	-	-	-	-
Proline	20.23	1.3	6 8.8	2 0.96	2 0.09	1.3	6 8.8	-	8.68	17.47	1.45
Glycine	26.09	-	1 5 1.5	21.31	18.49	-	1 5 1.5	55.4	15 .94	11.43	2.98
Alanine	9.12	13.56	6 6.8	6.32	5.58	13 .56	6 6.8	58.9	8.04	11.08	1.33
Valine	1.96	2.64	3 2.6	2.21	2.04	2.64	3 2.6	20.0	2.37	13.49	0.56
Methionine	1.35	2.51	2.07	0.94	1.23	2.51	2.07	21.3	2.61	20.57	0.48
Isoleucine	2.04	1.52	1 6.0	2.86	3.02	1.52	1 6.0	17.4	1.91	15.44	0.35
Leucine	3.	3.	2	4.	4.	3.	2	90.	4.	15.2	0.

	37	53	3.4	58	78	53	3.4	3	99	7	77
Tyrosine	1.35	0.9	6.83	1.65	2.53	0.9	6.83	62.8	1.11	26.2	0.40
Phenylalanine	1.83	1.84	8.21	3.39	5.19	1.84	8.21	62.6	2.61	23.18	0.64
Histidine	0.14	1.64	1.22	0.87	1.20	1.64	1.22	11.9	1.84	29.32	2.06
Hydroxyproline	-	0.82	-	-	-	0.82	-	-	-	-	-
Lysine	2.09	3.91	-	5.05	5.54	3.91	-	26.5	5.79	25.52	0.54
Arginine	5.14	6.07	-	14.64	17.21	6.07	-	215	7.28	27.52	2.76
Tryptophan	-	6.02	33.0	-	-	6.02	33.0	-	1.14	-	-
Cystine	6.58	0.62	2.85	4.97	5.01	0.62	2.85	-	3.17	-	0.05

Amino acids	cat fish (Yen et al., 2010)		ribbon fish (Nazeer, and	seela fish (Nazeer and Dee	sea breams (Akagunduz et al., 2014)			grass carp whole fi	grass carp Whol
	Skin soluble	Skin insoluble FPH			Scales	Bone Alk. Digestion	Bone HCL Digestion		
Aspartica cid /Aspergine	7.53	8.87	2.79	1.99	45	40	46	6.24	78.7
Threonine	3.04	3.91	3.09	3.47	24	25	25	2.98	-
Serine	3.11	3.40	0.94	2.63	46	47	47	3.03	33
Glutamic acid	11.68	11.80	4.56	5.61	70	70	71	9.05	93.4

Glutamine		-	-	-	-	-	-	-	-
Proline	9.49	5.58	5.01	5.87	113	112	108	3	455
Glycine	18.70	11.94	0.60	1.43	345	339	341	5.1	47.9
Alanine	8.94	6.56	1.06	1.25	123	123	121	3.86	40.3
Valine	3.67	5.39	2.81	2.81	17	18	19	2.79	35.7
Methionine	0.44	1.35	4.32	5.22	15	14	16	1.82	26.9
Isoleucine	2.67	4.73	3.01	9.71	5	7	8	2.75	36.5
Leucine	4.42	7.38	1.57	3.8	18	20	21	5.06	85.1
Tyrosine	1.77	3.74	16.09	9.53	4	4	3	2.69	41.1
Phenylalanine	2.61	4.69	15.36	7.41	14	14	14	2.72	54.1
Histidine	1.59	2.49	7.85	7.55	5	6	5	2.51	45.4
Hydroxyproline	0.70	0.66	-	-	72	72	3	-	-
Lysine	4.62	6.05	1.57	3.8	27	25	24	6.51	-
Arginine	8.81	8.43	13.38	11.6	50	50	49	28	27
Tryptophan	-	-	1.09	2.57	-	-	-	-	35.4
Cystine	-	-	2.96	4.96	1	2	2	23	-
Hydroxyl	-	-	-	-	6	7	6	-	5.8

PH, pepsin protein hydrolysate; PPH, pepsin + pancreatin protein hydrolysate; FPH, Fish protein hydrolysate.

Table 3: ACE- inhibitory activity of fish protein hydrolysates and its peptides

Fish specie	Fish parts	Enzymes	Purified peptides	Molecular weight	IC_{50} Value	References
Alaska Pollack, (*Theragra chalcogramma*)	Whole	Pepsin	Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala	1-3 kDa	14.7 µM	Je et al., 2006
Catfish (*Silariformes*)	Whole	Protamex®	-	<1 kDa	-	Theodore and Kristinsson 2007
Tuna (*Tunnini*)	Whole	Alcalase, neutrase, pepsin, papain, α-	Gly-Asp-Leu-Gly-	2 kDa	11.28 µM	Hoon et al., 2008
Goby fish *(Zosterisessor ophiocephalus)*	Whole	A21Proteases	1. Ala-Arg-Ser 2. Val-Val-Ala-Pro-	-	*1.36 mg/ml	Nasri et al., 2013
Jelly fish (*Rhopilemaes culentum*)	Whole	Pepsin and papain	Gly-Glu-Pro-Asp-Ala	2kDa	1.28 mg/ml	Xin et al., 2012
Lizard fish (*Synodus indicus*)	Whole	Neutral proteases	Gly-Met-Lys-Cys-Ala-Phe	5kDa	45.7 µM	Xiongdio et al., 2015
Boarfish (*Capros aper Linnaeus*)	Whole	Alcalase, papain and protease AP	-	3-10kDa	-	Hayes et al., 2016
Hound fish (*Caprosaper linnaeus*)	Viscera	Alkaline proteases extract	-	-	*75 µg/ml	Abdelhedi et al., 2016
Sardinelle (*Sardinella aurita*	Viscera	Alcalase α-Chymotryps	-	-	*1.2 mg/ml *0.81	Bougatef et al., 2008

Salmon (*Salmo salara)*	Skin	None (water)	1. Ala-Pro 2. Val-Arg	-	0.06mg/ml 0.33mg/ml	Gu et al., 2011
Chum Salmon (*Oncorhynchusketa keta*)	Skin	Trypsin	Gly-Leu-Pro-Leu-Asn-Leu-Pro	770Da	18.7 µM	Jung et al., 2014
Pacific cod fish *(Gadus microcephalus)*	Skin	Pepsin, papain, chymotrypsin, trypsin,	1. Gly-Ala-Ser-Ser-	662Da 436Da	6.9 µM 14.5 µM	Ngo et al., 2016
Nile tilapia (*Oreochromis niloticus)*	Skin	Alcalase	-	1.1kDa	*1.2 mg/ml	Thuanthong et al., 2017
Salmon (*Salmo salara)*	Skin	Alcalase and flavourzyme	1. Gly-Gly-Pro-Ala-Gly-Pro-Ala-Val 2. Gly-Pro-Val-Ala 3. Pro-Pro 4. Gly-Phe 5. Arg 6. Tyr	-	0.673mM 0.445mM 1.912mM 0.178mM 0.098mM 0.132mM	Neves et al., 2017
Ribbon fish (*Trichiurus haumela*)	Backbone	Acid proteases	Leu-Trp	317.25 Da	5.6 µM	Ping et al., 2014
Yellowfin sole (*Limanda aspera)*	Backbone	α-Chymotrypsin	Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu	5kDa	28.7µg/ml	Jung, et al., 2006

Shark (*Selachimorpha*)	Muscle	Proteases SM98011	1. Cys-Phe 2. Glu-Tyr 3. Phe-Glu	-	1.96 µM 2.68 µM 1.45 µM	Wu et al., 2008
Carp fish (*Ctenopharyngodon idella*)	Muscle	Pepsin	1. Tyr-Asn-Leu-Lys-Glu-	-	1.35µM 3.42µM	Zhang et al., 2017
Pipefish *(Syngnathuss chlegeli)*	Muscle	Alcalase	1. Thr-Phe-Pro-His-Gly-Pro 2. His-Trp-Thr-Thr-Gln-Arg	714Da 917 Da	0.62 mg/ml 1.44 mg/ml	Wijesekara et al., 2011
Sardinelle (*Sardina pilchardus)*	Muscle	A26 and An6 proteases	1. Asn-Val-Pro-Val-Tyr-Glu-Tyr 2. Ile-Thr-Ala-Leu-Ala-Pro-Ser-Thr-Met	-	0.21mM 0.23mM	Jemil et al., 2017
Sardine (*Sardina pilchardus)*	Heads and viscera	Alcalase	-	152-861Da	*1.16mg/ml	Martinez-Alvarez et al., 2016
Tuna *(Thunnus thynnus)*	Heads Muscle debris and viscera	Alcalase	-	170-1107Da	*0.27mg/ml *0.24mg/ml	Martinez-Alvarez et al., 2016
Cuttle fish *(Sepia officinalis)*	Muscle	A21 proteases Cuttlefish proteases	1. Val-Glu-Leu-Tyr-Pro	-	5.22µM 18.2µM 14.41µM	Balti et al., 2015

			2. Ala-Phe-Val-Gly-Tyr-Val-Leu-Pro 3. Glu-Lys-Ser-Tyr-Glu-Leu-Pro			
Horse mackerel (*Trachurus trchurus*)	Whole	Subtilisin Pancreatic trypsin	1. His-Leu-Ala-Leu-Thr 2. Met-Trp-His-Asn-Ala-His 3. Arg-Glu-Leu-Ala-Gly-Pro 4. Glu-Leu-Ser-Ala-Pro	553Da 794Da 640Da 515Da	5.11 µM - 6.24 µM 7.08 µM	Gracia-Moreno et al., 2015
Small-spotted catshark (*Scyliorhinidae*)	Whole	Subtilisin pancreatic trypsin	1. Glu-Leu-Val-Gly-Val 2. Leu-Val-Ala-Pro-Ala-Asn 3. Tyr-Leu-Gly-Trp 4. Val-Ala-	515Da 583Da 537Da 563Da	0.51 µM 0.90 µM 0.09 µM 0.44 µM	Gracia-Moreno et al., 2015

			Met-Pro-Phe			
Trevally fish (*Pseudocaranx* sp.)	Whole	Bromelain	1. Ala-Arg 2. Ala-Val 3. Ala-Pro-Gln-Arg	245Da 188Da 471Da	448 µM 371 µM 190 µM	Salampessy et al., 2015
Leatherjacket fish (*Meuchenia* sp.)	Whole	Papain, Bromelain Flavourzyme	1. Glu-Pro-Leu-Trp-Val 2. Asp-Pro-His-Ile 3. Ala-Glu-Arg 4. Glu-Gln-Ile-Asp-Asn-Leu-Glu 5. Tyr-Asp-Asp-Met-Glu	619Da 480Da 374Da 858Da 694Da	0.05mg/ml 0.02mg/ml 0.01mg/ml 0.24mg/ml 0.01mg/ml	Salampessy et al., 2017
Jumbo squid (*Dosidicus gigas*)	Tunic skin	Esperase	-	<1kDa	*0.096mg/ml	Mosquera et al., 2015
Sea bream (*Sparidae*)	Bones, scales	Alcalase and esperase	-	<3kDa	*60 µg/ml	Akagundaz et al., 2014
Carp fish (*Cirrhinus mrigala*)	Muscle	Papain	-	0.339-7kDa	*1.15-1.53mg/ml	Elavarasan et al., 2016
Cuttle fish (*Sepiida*)	Industrial effluent	Alcalase	1. Pro-Glu-GLn-Ser-Ile-Arg-Pro- 2. Thr-Arg-	755Da 705Da 755Da	*1.92mg/ml	Amado et al., 2014

			3. Ala-Ala-Cys-Pro Ser-Pro-Ser-Ile-Ala-Pro-Ala-Leu			
Flounder fish (*Daralichthys olivaceus*)	Muscle	Pepsin, papain, trypsin, kojizyme	1. Met-Glu-Val-Phe-Val-Pro 2. Val-Ser-Gln-Leu-Thr-Arg	721Da 703Da	79 µM 105 µM	Ko et al., 2016
Tilapia (*Oreochromis niloticus)*	Muscle	SK1-3-7 protenases	1. Met-Cys-Ser 2. Leu-Leu-Pro 3. Pro-Glu-Pro 4. Arg-His-Leu	340Da 341Da 341Da 345Da	0.29 µM 0.41 µM 0.47 µM 1.23 µM	Toopcha m et al., 2017
Goby fish (*Zosterissessor ophiocephalus*	Muscle	I) Alkaline protease extract from 1. Smooth hound 2. Grey trigger fish 3. Golden mullet 4. Goby II) Bovine trypsin	-	-	*0.833mg/ml *0.73mg/ml *0.13mg/ml *0.9mg/ml *1.05mg/ml	Nasri et al., 2013
Lizard fish (*Saurida elongate*)	Muscle	Neutral proteases	Arg-Tyr-Arg-Pro	<5kDa	52 µM	Sun et al., 2017

Salmon (*Salmon salar*)	Muscle	Human and porcine gastrointestinal enzymes	1. Ile-Trp 2. Ile-Tyr 3. Thr-Val-Tyr 4. Val-Pro-Trp 5. Val-Tyr	-	*0.91-1.04mg/ml	Darewiez et al., 2014
Tilapia (*Oreochromis niloticus)*	Muscle	Sk1-3-7 protenases	Met-Ile-Leu-Leu-Leu-Phe-Arg	905Da	0.1mg/ml	Toopchiam et al., 2015
Snakehead fish (*Channidae*)	Muscle	Alcalase, Flavorzyme, papain, protenases	1. Leu-Tyr-Pro-Pro 2. Tyr-Ser-Met-Tyr-Pro-Pro	-	1.3 µM 2.8 µM	Ghassem et al., 2014
Thornback ray fish (*Raja clavata*)	Muscle	A26 proteases	1. Phe-Glu-Pro-Ser-Phe 2. Leu-Lys-Tyr-Pro-Ile	-	12.50 µM 27.07 µM	Lassoued et al., 2016
Basa fish (*Pangsius bocourti*)	Skin	Protenases, alcalase, neutrase, trypsin, pepsin	-	-	*1.417mg/ml	Zhang et al., 2016
Mackerel (*Scomber scombrus)*	Skin	Pepsin	1. Phe-Gly-Asn 2. His-Gly-Pro-Leu	337Da 423Da	-	Khiari et al., 2014

Squid (*Teuthida*)	Tunic skin	Pepsin Esperase	Gly-Arg-Gly-Ser-Val-Pro-Ala-Hyp-Gly-Pro	<1kDa	478 µM	Aleman et al., 2013
Lizard fish (*Saurida elongate*)	Muscle	Nuetral protease	Arg-Val-Cys-Leu-Pro	-	175µM	Wu et al., 2015
Cape hake (*Merluccius capensis*)	Industrial waste	Cod protein hydrolysate	-	-	*1mg/ml	Pires et al., 2015
Skate (*Okamejei kenojei*)	Skin	Alcalase and proteases	Leu-Gly-Pro-Leu-Gly-His-Gln Met-Val-Gly-Ser-Ala-Pro-Gly-Val-Leu	720Da 829Da	4.22 µM 3.09 µM	Ngo et al., 2015
Loach (*Misgurnus anguillicaudatus*)	Muscle	Bromelain	Ala-His-Leu-Leu	-	-	Li et al., 2016

Asp, Aspartic acid; Asp, Aspergine; Thr, Threonine; Ser, Serine; Glu, Glutamicacid; Gln, Glutamine; Pro, Proline; Gly, Glycine; Aln, Alanine; Val, Valine; Met, Methionine; Il Isoleucine; Leu, Leucine; Tyr, Tyrosine; Phe, Phenylalanine; His, Histidine; Lys, Lysine, Arg, Arginine; Try, Tryptophan; Cys, Cystine.
* indicates IC_{50} value of the crude FPH