MARINE FUNCTIONAL PROTEINS & THEIR APPLICATION

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Introduction

In today's scenario, a large portion of the global population is very much aware of health benefits one can achieve through consumption of aquatic food products. Particularly, fish and shellfish are highly nutritious and delicious. The demand for fish is ever increasing. On the other hand, aquatic animals like fish and shellfish are highly perishable compared to meat from land animals due to near neutral post mortem pH, low glycogen reservoir, low connective tissue content and high moisture content. Immediately after harvesting of fish (immediately after death), it undergoes various bio-chemical and microbiological changes which lead to spoilage. Functional proteins can be obtained from edible portion of meat from aquatic animals as well as waste from the aquatic food waste. Hence, fish is essentially processed and preserved to make the fish available in edible condition. As a result of processing, a greater portion of raw material is discarded as waste which is biochemically equivalent to edible portion. This chapter doesn't include the content or process details for obtaining proteins from aquatic plants, planktons and microalgae.

Fish muscle proteins

The proteins in fish muscle can be classified into three groups as given by Huss (1995).

1. *Sarcoplasmic proteins* (myoalbumin, globulin and enzymes) which are soluble in neutral salt solutions of low ionic strength (< 0.15 M). This fraction constitutes 25-30 percent of the total proteins. About 100 different proteins are known to be present in the sarcoplasmic fraction.
2. *Structural proteins* (actin, myosin, tropomyosin and actomyosin) which constitute 60-70 % of the total protein content. These proteins are soluble in neutral salt solutions of fairly high ionic strength (0.5 M and above)

3. *Stroma proteins*, which constitute approximately 3 % of the protein in teleostei and about 10 % in elasmobranchii (compared with 17 % in mammals). They include connective tissue, collagen and elastin.

Sarcoplasmic proteins

The sarcoplasmic proteins consist mostly enzymes and include proteinases, peptidases and cathepsins. The cathepsins are a group of muscle proteases and can cause softening of the fish tissue (Ladrat *et al*., 2003). Apart from enzymes, pigments such as myoglobin and a low molecular weight protein parvalbumin are present in sarcoplasm which can significantly influence the quality of fish meat and FPH. Myoglobin is a conjugated protein which has high binding co-efficient with oxygen and acts as a storage means of oxygen (Baron and Andersen, 2002). Depending on the oxidation state of iron atom, the colour of the product including FPH will vary. Parvalbumin is a small protein with a molecular weight of 12 kDa and is involved in calcium signale either for binding and release (Taylor *et al*., 2004). There are reports that the allergic reactions are caused in some populations of human by consumption of parvalbumin.

Myofibrillar proteins

The myofibrillar protein comprises of more than 15 different fractions and myosin is the abundant molecule (Sikorski, 1994). The different myofibrillar protein fractions with their molecular mass and number of subunits are given in Table 1. Myosin constitutes nearly 55 % of the total myofibrillar proteins (Murray *et al*., 1993). The myosin molecule is relatively large in size with a molecular weight of 4.8 - 5.0 x 10^5 Da (Rayment *et al*., 1993). The number of subunits in myosin molecule vary from 4 - 6. The two polypeptides in the myosin molecule are highly coiled and terminate into the globular head. These two are referred as myosin heavy chains (MHC). The other subunits are small in size and referred as light chains (Wang *et al*., 2009). The average number of residues in myosin molecule is 2300 - 2700. The primary structure of different myosins from the muscle system has been determined by protein sequencing and cDNA or genomic DNA cloning. The sequence showed considerably high homology between carp, chicken and rabbit myosin. The relative instability of fish myosin has been attributed to the nature of residue in S2 fraction. The amino acid at position 1078 in pollock and carp myosin is serine which has hydrophilic side chain while that in rabbit and chicken is leucin which has hydrophobic side chain (Ojima *et al*., 1998). It has been established that native myosin molecule do not have disulfide bridge despite 40 free sulfhydryl residues found in the molecule. The globular head possess

binding site for nucleotide and for actin molecule. In myosin head three different domains have been identified together constitute the molecular mass of 95 kDa. It consists of 25 kDa N-terminal domains, a 50 kDa central domain and a 20 kDa C-terminal domain. The 25 kDa and 50 kDa domains form the ATP binding site while 50 kDa and 20 kDa domains form actin binding site. The two light chains binding sites are located at 20 kDa domain and they wrap around 20 kDa domain.

Actin is ubiquitous in muscle tissue and is the major constituent of thin filaments. It makes up to 25 % of myofibrillar protein by weight (Murray *et al*., 1993). Actin exists in two forms - one is globular (G-actin) and another one is fibrous (F-actin). F-actin is formed by polymerization of G-actin. G-actin consists of a single polypeptide chain, a nucleotide (ATP) and a divalent cation bound to a specific site. The average molecular weight of actin is 42 kDa and consists of 375 amino acid residues among which 5 are free sulfhydryl residues (Dominguez and Holmes, 2011).

Tropomyosin is a regulatory protein in the myofibril (Xiong, 1997). It is a filamentous molecule composed of a coiled coil of two α-helices. The two α-helices are dissimilar, designated as α- and β-tropomyosin with molecular weight of 34 and 36 kDa respectively. Each subunit α-helix usually consists of 284 amino acid residues (Inoue *et al*., 1998).

Troponin is a complex of three polypeptide chains (Tn C-18 kDa; Tn I-24 kDa; Tn T-37 kDa). The number of amino acid residues in TnC, TnI and TnT is 159, 179 and 259 respectively (Stryer, 1995, Inoue *et al*., 1998; Nishita *et al*., 1994).

The other myofibrillar proteins include α actinin which constitutes the Z-disk, the C-protein associated with myosin, M-line proteins (Myomesin, M-protein and creatine kinase) found in the M-line region and H-and X-proteins associated with myosin at discrete sites on the surface of the thick filaments (Xiong, 1997). The FPH prepared from fish meat / processing waste has to take into consideration the properties and nature of residues in major protein fractions which will have a bearing on the end product.

Stroma proteins

The stroma protein mainly comprises of connective tissue and collagen. The collagen content of fish is lower as compared to mammalian counterpart (Bremner, 1992). Apart from providing the structural integrity to the muscle fiber, collagen is a major component of skin and bones. Collagen is a multimeric protein with 3 subunits. The 3 polypeptide chains are highly coiled and lack α-helical structure. The proportion of proline and hydroxyl proline is about 10 % of total amino acid residues together referred as imino acids. The special feature of collagen is occurrence

of glycine at every 3rd residue. The technological significance of collagen during processing is related to the texture. During chilled storage of blue grenadier (*Macruronus novaezelandiae*), it was observed that the attachment between muscle fibers and myocommata and the whole sarcolemma was degraded leading to detachment of muscle fiber (Bremner and Hallett, 1985; Hallett and Bremner, 1988). A similar degradation was observed with king salmon (*Oncorhynchus tshawytscha*) (Fletcher *et al*., 1997), Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*) (Ofstad *et al.*, 1996). The intermolecular cross-links in collagen are thought to be responsible for the stability, physical strength and mechanical properties of the connective tissue and other components of the extracellular matrix (Bracho and Haard, 1995). Breakdown of the connective tissue by endogenous collagenases may lead to undesirable textural changes in the fish.

Secondary raw material

Aquatic food processing discards are now called as secondary raw material because of their potential for the production of high value products. For any country, to develop a systematic way to utilize or to set up an industry, the information on amount of waste generated would be the first aspect to be searched. Unfortunately, even in well developed countries, the data on waste generation from fish processing sector is not available, due to the complexity in obtaining such information. The available data are derived from the information on export quantity. However, it is essential to have information part wise, as many of the high value ingredients are derived from the specific parts (organs). The properties of derived high value products depend on the parts from which they are derived. For example, the properties of gelatin from fish skin, scale and bone are different.

Protein content in secondary raw material

The discards from fish/shellfish contain protein in the range of 9-27% depends on the waste parts. The tissue proteins for example the meat from head and filleting frames contains major muscle protein fractions like myosin, actin, troponin, tropomyosin etc. The skin, scale and bone contains the protein namely collagen (an integral protein moiety of connective tissues). Shrimp shell waste contains carotenoproteins.

Table 4. Protein content in major fish waste parts

	Waste Parts	Protein (%)
1.	Head	11-13
2.	Back-bone/ frame	10-15
3.	Cut-offs	12-22
4.	Skin	8-12
5.	Milt	14-27
6.	Viscera	9-23
7.	Shrimp head waste	9-14%

(Source: Rustard, 2007**)**

Proteins from secondary raw material and the possible industrial products

Fish processing discards are rich in fish muscle proteins (Myosin, actin troponin, tropomyosin etc.), connective tissue proteins (Collagen and its derivative gelatin), fish enzymes, hemoproteins and carotenoproteins. The relevant industrial products which exploit the above mentioned proteins are fish protein concentrate, surimi from frame meat, fish meal, shrimp head meal, squid meal, dried fish scale and dried fish skin.

Table 5. The protein components from secondary raw material and the relevant possible industrial products

Proteins from secondary raw material	Protein rich industrial products from secondary raw material
• Fish muscle proteins (Myosin, actin troponin, tropomyosin) • Collagen • Gelatin • Fish enzymes • Hemoproteins • Carotenoproteins	• Fish protein concentrate/fish protein powder • Surimi • Fish meal • Shrimp head meal • Clam meal • Squid meal • Dried fish scale • Dried fish skin

Fish protein concentrate

Fish protein powder (FPP) is a dried fish product, meant for human consumption, in which the protein is more concentrated than in the original fish flesh. Different methods for the separation of meat from fish are employed, such as washing meat with water for two to 3 cycles and concentrating, solubilization of muscle by pH adjustment and iso-electric precipitation, solvent extraction to method to remove the fat, cooking and drying, and a combination of various methods. The raw material such as fish filleting frames, head waste, tuna red meat and belly flaps can be used to produce fish protein concentrate

Earlier studies conducted on rat have shown that fish proteins have greater cholesterol lowering ability (Ammu et al., 1989) and can protect the animal against lipid peroxidation. Fish protein reduces serum cholesterol, triglycerides and free fatty acids and increases the proportion of HDL cholesterol. In general, protein supplements claims to help weight loss and muscle building. Fish protein supplement have shown beneficial effects on blood levels of glucose and LDL-cholesterol as well as glucose tolerance and nutritional composition of body in overweight adults (Vikoren et al., 2013). In another study, dietary scallop protein completely prevented high-fat, high-sucrose-induced obesity whilst maintaining content of lean body mass and improving the lipid profile of plasma in male C57BL/6J mice (Tastesan et al., 2014).

Fish Collagen

Collagen is a structural protein found mainly in the skin and bones of all animals. Collagen is the most abundant protein originating from the animal source, comprising approximately 30% of total animal protein. It is composed of three α-chains which are intertwined to form a triple-helix. It is present in the connective tissue matrix that makes the framework of skin, bones and joints, cornea, blood ducts, and the placenta. There are many types of collagen, but 90% of our body's collagen protein is Type-I collagen. It is found to be rich in amino acids such as glycine, valine, alanine, proline and hydroxyproline (Burghagen, 1999). Glycine constitutes one third of the total amino acid content of collagen followed by hydroxyproline and proline, which account for another one-third. Owing to this structural uniqueness of collagen molecule, there is increasing interest for the direct consumption of collagen in the form of their easily digestible derivatives. Worldwide, this interest has been taken-up by the nutraceutical industry, especially in developing countries.

Currently, collagen is used in many pharmaceutical and cosmetic products, due to its structural role and better compatibility with human body. It is commonly used in the cosmetic

industry for the production of skin lotions as it forms a superior protective film to soothen and hydrate the skin. Such potential of collagen has tremendous bearing on anti-aging treatment. Apart from that, collagen has a wide range of applications in the field of cosmetic and burn surgery, especially as dermal fillers in the reconstruction of skin and bone. Collagen gels have potential clinical importance in the preparation of 'artificial skin' used in treating major wounds. Injectable collagen hydrogels have been successfully used for soft-tissue augmentation, drug delivery carriers and hard-tissue augmentation. Microfibrous collagen sheets are used as promising drug carriers for the treatment of cancer. It is also an essential component in diverse orthopedic and dental treatments. Further, collagen is recently projected as a joint mobility supplement.

Fish Gelatin

Gelatin is a soluble polypeptide obtained by denaturing the insoluble collagen. Procedures to derive gelatin involve the breakdown of cross-linkages existing between the polypeptide chains of collagen along with some amount of breakage of intra-polypeptide chain bonds. Tissues that contain collagen are subjected to mild degradative processes, i.e., treatment using alkali or acid followed or accompanied by heating in the presence of water, the systematic fibrous structure of collagen is broken down irreversibly and gelatin is obtained. It is the only protein based food material that gels and melts reversibly below the human body temperature (37°C). Gelatin possesses unique and outstanding functional properties and can be obtained in reasonable cost, make it one of the most widely used food and pharmaceutical ingredient.

Fish skins and bones can be utilized to produce gelatin, thus contributing to solve the problems of waste disposal with the advantage of value addition. The main drawback of the fish gelatins are the gels based on them tend to be less stable and have inferior rheological properties compared to mammalian gelatins. It may be noted that fish gelatin has its own unique properties like better release of a product’s aroma and flavor with less inherent off-flavor and off-odor than a commercial pork gelatin, which offer new opportunities to product developers.

Fish enzymes

Fish visceral waste can serve as a source of large amount of enzymes which have potential applications in different sector starting from laundry application to pharmaceutical applications (Simpson and Haard, 1987). The nature of fish visceral enzymes is different from the enzymes found in the digestive system of terrestrial animals. Hence, they can be exploited for certain distinct applications. Fish pepsins can act even at low temperature and higher pH optimum than

the pepsins from terrestrial source. Moreover, fish pepsins do not undergo autolysis at low pH (Raa, 1990). The differences in the properties of pepsins from fish and other sources could be attributed to the difference in the sequence and composition of aminoacids (Gildberg and 0verbj, 1990). Fish enzymes can be used as processing aids in the following applications

- Protein hydrolysates production
- In production of caviar from a variety of fish species
- for removal of squid skin
- for cleaning of scallop
- for descaling of fish
- coagulation of milk
- Cheese production

Hemoproteins

Hemoproteins are complex proteins, composed of a protein molecule and a non-protein compound (prosthetic group). Hemoglobin and myoglobin belongs to the category of hemoproteins involves in transport of oxygen in the blood and tissues of animals, respectively. The heme portion can be recovered from blood as well as muscles discards. The recovered material may be used iron supplement or as a chemical substrate for production of the cooked cured-meat pigment. During the production of hydrolyzates from meat, hemin could be recovered as by-product.

Carotenoproteins

Carotenoproteins and carotenoids are other classes of compounds found in the flesh and skin of fishes and in the exoskeleton of shellfish. They are not synthesized in their body. They are acquired through their food chain (Haard, 1992). Similar to hemoproteins, Carotenoids are also composed of a protein moiety and a non-protein prosthetic group. Isolation of carotenoproteins and carotenoids from shellfish processing discards has been reported (Long and Haard, 1988). Inclusion of caratenoids pigments in feed formulations of some of the aquacultured fishes and ornamental fishes shows the importance of these compounds in industrial applications (Shahidi et al., 1993).

Protein derivatives from secondary raw material

Fish protein hydrolysates (Bioactive peptides)

Apart from being highly nutritious, fish muscle proteins can be made use for preparing fish protein hydrolysates which comprises of bioactive peptides with valuable nutraceutical and pharmaceutical potentials. Fish protein hydrolysates (FPH) are the mixture of amino acids and peptides obtained by digesting proteins from fish meat or fish processing waste with proteases.The size of these peptides may range from 2 to 20 amino acid residues with the molecular masses of <6000 Da and are highly bioactive. The food derived peptides can be used as functional food ingredients or as nutraceuticals to benefit the human health and prevent disease. In this context, large pharmaceutical companies are more interested to invest in bioactive peptide research to open therapeutic prospects.

Application of fish protein hydrolysates

Nutritional application

The proximate composition of fish protein hydrolysate would vary with the raw material (head, bone, skin, viscera), type of process, type of drying, extent of hydrolysis and any other pre-treatment of raw material. The chemical composition of food materials has an important role on human health in supply of essential nutrients for maintaining prosperous health. Chemical composition of fish protein hydrolysates is important in nutrition perspective of human health.

Table 6. Proximate composition of fish protein hydrolysate

Waste Parts	**Protein (%)**
Moisture	< 10 %
Protein	60-90 %
Fat	<5 %
Ash	0.45-27%

(Source; Chalamaiah et al., 2010)

Amino acid composition of protein hydrolysates from different raw material produced using different enzyme source under different hydrolysis conditions expected to have variation. In general, required essential amino acids are abundant in FPH with richness in glutamic and aspartic acid content. FPH do also have non-essential amino acids. Presence of aromatic amino acid in fish frame protein hydrolysates has been reported. Studies have clearly shown that FPH from fish meat/fish waste could be an ideal source of essential amino acids (Chalamaiah et al., 2010).

Nutraceutical applications

There are fish protein hydrolysate products/peptides specifically marketed as health supplements in developed countries. These products are proven to have specific health role other than the nutritional benefit. Protein hyd5rolysates or peptides present in the hydrolysate have demonstrated to have antioxidant, anti-obesity, immune modulation, anti-coagulation, anti-microbial, anticancer and antihypertension etc. (Elavarasan et al., 2014; and Elavarasan et al., 2016).

Table 7. Commercially marketed fish protein hdyrolysate products as Nutraceuticals

Product brand name	Particulars	Nutraceutical applications	Country
PROTIZEN®	Produced by enzymatic hydrolysis of white fish proteins	It is “mood food” and dietary supplement to fight against stress and its symptoms (weight disorders<comma> work pressure<comma> sleep troubles<comma> concentration difficulties and mood troubles).	UK
Amizate®	Produced from Atlantic salmon fish proteins by autolysis	Sports nutrition (supports the body’s muscle anabolism and metabolic recovery).	North America
Nutripeptin®	Manufactured by enzymatic hydrolysis of Cod fish fillet/muscle protein	It helps in the blood glucose stabilization<comma> and weight management.	UK and USA

Seacure®	Prepared by hydrolyzing deep ocean white fish proteins	Dietary supplement helps to support the cells in the gastrointestinal tract and regulate bowel functions.	US and Canada
Vasotensin®	Produced from Bonito (*Sarda orientalis*) by thermolysin hydrolysis	It supports healthy vascular function for optimal blood flow and healthy blood pressure levels.	US and Japan
LIQUAMEN®	Prepared from *Molva molva* by autolysis	Dietary supplement that helps in reducing oxidative stress<comma> lowering glycemic index and anti-stress.	UK
Stabilium® 200	Prepared from *Molva dypterygia* by autolysis	Supports the body's response to stress and provides nutritional support for memory and cognitive function.	UK
PEPTACE®	Produced from Bonito (*Sarda orientalis*) by thermolysin hydrolysis	It lowers the blood pressure by inhibiting ACE enzyme.	US and Japan
SEAGEST®	Prepared by hydrolyzing deep ocean white fish proteins	It supports the structure of the intestinal lining and promotes intestinal health.	US
MOLVAL®	Produced from North Atlantic fish *Molva molva* by enzymatic hydrolysis	Dietary supplement recommended for cholesterol equilibrium<comma> stress control and promotes good cardiovascular health.	UK

(Source: Chalamaiah et al., 2010)

Fish protein hydrolysate as a functional ingredient

Fish protein hydrolysates are soluble in wide range of pH which is an ideal characteristic helps to use in wide range of products. Protein hydrolysates have improved water holding, oil binding, emulsifying and foaming properties. However, the key factor which determine the functional properties is degree of hydrolysis. In general, extensive hydrolysis leads to loss of functionality. There is a critical degree of hydrolysis at which protein hydrolysates should be prepared with reference to particular function to be used as a functional ingredient (Elavarasan et al., 2016; Gajanan et al., 2017).

Collagen peptide/gelatin hydrolysate

Collagen peptide is alternatively known as 'collagen hydrolysate', 'gelatin hydrolysate' and 'hydrolysed collagen'. Since collagen and gelatin are high molecular weight proteins of approximately 300 kDa, it is difficult for digestion and hence becomes unavailable to human body for their biological functions. Consequently, in recent years, much attention has been paid to the development of small molecular weight peptides from the native collagen with improved biological activities. This can be achieved by the process of hydrolysis in which the native collagen/gelatin molecules are cleaved to small fragments. The hydrolysis process leads to formation of fragmenting from the collagen of about 300 kDa to small peptides having an average molecular weight of less than 5 kDa. The visible consequence of this hydrolytic transformation is the complete dissolution of resultant peptide mixture in cold water, which further widens the application prospects of collagen peptide.

Small peptides are desirable for nutraceutical and pharmaceutical applications, whereas large peptides are desirable for the functional modification of food products. Standardisation of collagen production technology is a stepping stone in the nutraceutical and health food industry. From a nutritional perspective, peptides are more bioavailable than proteins or free amino acids and at the same time, less allergenic than their native proteins (Otani et al., 1990). Apart from that collagen peptides are shown to promote the absorption of vitamins and minerals. Hence, recently combined formulations of collagen peptide with minerals and vitamins are coming up in the market. Apart from their nutritional benefits, bioactive collagen peptides possess a wide range of physiological functions including antihypertensive, antioxidative, anticancer, immunomodulatory, antimicrobial, mineral binding, antithrombotic and hypocholesterolemic effects (Gomez-Guillen et al., 2011).

Enzymatically hydrolyzed collagen have shown better biological activities compared to the peptides derived from fish muscle protein with antioxidants and antihypertensive agents.

Table 8. The protein derivatives from secondary raw material and the possible industrial products

Protein derivatives from secondary raw material	Protein derivatives based industrial products from secondary raw material
• Fish protein hydrolysate • Collagen peptides • Gelatin hydrolysate	• Fish silage • Flavorings • Collagen peptides • Gelatin hydrolysate • Fish protein hydrolysate • Shrimp protein hydrolysate • Fish waste paste • Cuttlefish and squid by-products paste

Conclusion

Globally, the aquatic food waste (secondary raw material) has been identified as source of high value functional ingredients. On the other hand current exploitation of aquatic food waste is happening as high volume low value products for example fish silage, fish meal, squid meal, shrimp head meal etc. The major high value protein based product from fish waste is collagen and its derivatives. The way the fish waste utilized in India needs a rattled shift in order to realize the full potential of seafood processing waste generated in India.

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