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Extraction and Purification of Bioactive Peptides from Tilapia (*Oreochromis*) Waste

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Abstract

In the fisheries industry, compared to other food industries, a large volume of by-products such as bone, skin, and viscera are produced, which are costly to treat and dispose of in the environment. The rapid growth of tilapia and its marketability have also been considered. The value of proteins as the main source of amino acids in human health has been proven. In addition to their nutritional value, proteins have biological functions. These proteins show their functions through bioactive peptides. Production methods of bio peptides affect their biological activity. The articles showed that the use of enzymatic hydrolysis method for therapeutic applications makes them more stable than fermentation and solvent extraction methods and is a safer method than the other two methods. Bioactive peptides have physiological effects such as antihypertensive, antioxidant, antimicrobial, anti-cancer, anti-diabetic, etc. and have many applications in the food, pharmaceutical and cosmetic industries and can be added to the food industry. The purpose of this article is to review the extraction of bioactive peptides from fish waste.

Keywords: Aquatic, Bioactive peptides, Biotechnology, Tilapia, Wastes.

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Introduction

About 70 percent of the Earth's surface is water-covered. The biodiversity in aquatic ecosystems is considerably greater than the land surface and it houses almost 70% of all living organisms. Marine organisms are recognized as a valuable source of nutrients and functional compounds, so the marine environment is considered as a rich source of natural products with wide therapeutic applications (1). Seafood wastes are valuable natural resources that have a wide range of functional characteristics, so they are potential materials for the medical, biological and food-drug industries. About 78% of fishing in developing and developed countries is allocated to human food and about 21% is disposed as food processing industry waste (2). Processing leads to the production of large amounts of biomass from fish waste. (E.g. skin, bones and fins are discarded every year. About 3.7 million tons / year) (3).

Bioactive peptides which derived from marine organisms have a variety of activities such as immune regulatory, antimicrobial, antioxidant, anticoagulant, hypocholesterolemic and anti-hypertensive effects (4,5).

In past few years, consumers' attention to the relationship between food and health has increased. Which has led the food industry to seek to produce products that prevent diseases. In this regard, there is a great demand from consumers to add beneficial and healthy compounds to food. Healthy foods promote consumer health (6).

In addition to their nutritional value, bioactive peptides have a physiological effect on the human body in terms of providing amino acids, so they have been introduced as functional food (7,8).

1- Tilapia

Tilapia is a member of the cichlid family and belongs to the order of perch, which has a rectangular body with small scales. The main habitat of this fish is Kenya in East Africa. This fish is native to Africa, the Mediterranean and the Middle East. Although there are about 80 species of tilapia, but only a few of them (12 species) have been used in the aquaculture industry (9,10). This fish is the second farmed fish after carp and it can be easily grown and propagated at a low cost. According to available reports, the production and consumption of this fish has increasing all over the world (10). More than 100 countries around the world are currently producing tilapia (FAO, 2010) Most of them are produced in Asian countries. China is the largest producer of tilapia in the world (11).

This fish is popular in many parts of the world due to its ease of reproduction and breeding and favorable marketing characteristics. In addition to the above, these items can also be mentioned: fast growth capacity, ability to tolerate different environmental conditions and its changes in terms of temperature, salinity and oxygen, resistance to stress and disease, fast reproduction and consume food at low nutritional levels such as algae, plankton, detritus and also agricultural waste (12). Tolerance of unfavorable water quality and ability to grow in salty and fresh water. The negative biologically aspects of breeding this fish include premature maturation and intolerance to low water temperatures (13).

The activity and nourishment of Tilapia decreases at the temperature below 20ºC and stops completely at a temperature below 16ºC. The consumption of Tilapia is growing rapidly worldwide (14).

The most important species of tilapia:

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1- Blue tilapia (*Oreochromis aureus*)

2- Nile tilapia (*Oreochromis nilotius*)

3- Mozambican tilapia (*Oreochromis mossambicus*)

4- Wami Tilapia (*Oreochromis urolepis* hornorum)

5. Red tilapia (*Oreochromis sp*.)

The most well-known species of this group of fish are indigo, blue, zebra and Mozambique tilapia. The Nile tilapia species has a dorsal fin with sharp and long spines, a broad shape and a caudal fin with distinct vertical stripes (10). Health consumption of tilapia is one of the ambiguities for not introducing and developing it in our country, however this fish contains the necessary nutritional standards and high nutritional value. Tilapia meat has a remarkable variety and favorable ratio of unsaturated fatty acids. It's also a good source of protein, calories, calcium, iron and phosphorus. The food and drug administration and the US environmental protection agency have recommended tilapia as one of the most suitable aquatic products for pregnant and lactating women and children (15). In addition to being a rich source of proteins and bioactive peptides, aquatic animals are also used as raw materials to produce physiologically important peptides for humans (7).

Aquaculture and waste from aquatic processing can also be used as an abundant and inexpensive resource to produce bioactive peptides. Table 1. shows the protein contents and yields of tilapia samples (16).

Table1. Crude protein content and yield of tilapia (17**)**.

Sample	Protein content (%w/w)	Yield (%w/w)
frame	31.17±0.75	NA
frame protein isolate	82.06±0.04	11.93±088
skins	80.75±0.97	NA
skin protein isolate	89.47±0.86	23.04±4042

NA; not available

a; Crude protein content was determined by micro-Kjeldahl method with triplicate measurement on dry basis.

b; Yields were calculated on the basis of the protein weights of tilapia frame and skin protein isolates against the tilapia frame and skin on dry basis.

2- Fish waste

Large amounts of waste from the aquaculture industry are discarded without any effort to make them efficient. Many seafood producers are not able to dump their waste directly into the sea due to environmental issues and have to pay a lot of money to refine these materials, so finding a suitable method as an alternative to disposing of these materials is essential. It should be noted that the wastes of the fisheries industry are rich in protein and fat, which accelerates corruption in them. If these biological compounds are used properly, they will reduce the environmental pollution caused by their disposal and will also lead to the production of high value-added products. The main by-products of aquatic processing industries, includes viscera, skin, scales, spine and trunk bones. Aquatic animals have a special nutritional status due to having important

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substances such as proteins, minerals, and unsaturated fatty acids. In recent decades, due to increasing population growth and malnutrition in many parts of the world, aquatic animals can be considered as a rich source of protein. During fish processing, large amounts of waste products, including water and solids, are produced and used depending on the process. These waste products will usually involve a mixture of different parts of the head, skin, bones and other parts of the animal's body. About 32% of aquatic processing waste includes bone and skin, which are rich in collagen. Hydrolyzed fish proteins have a wide range of applications. These substances are considered as suitable substances for the treatment of cancer due to their bioactive peptides, chondroitin sulfate and antioxidant properties. Also, due to the short peptide chain, they have high digestibility and can be used as a protein supplement in human, livestock and aquatic nutrition (17). The prevalence of bovine insanity in recent years has prompted researchers to replace hydrolyzed marine proteins with animal proteins and introduce them to the public (7).

3- History of hydrolyzed protein

Hydrolysis of dietary proteins has a long history, mainly in the case of vegetable proteins and milk. The first commercial hydrolyzed protein was introduced in the 1940s (18). In the 1960s, a lot of research was done to obtain cheap, nutritious protein sources to feed the growing human population as well as animals, with much attention being paid to waste (6). The main purpose of hydrolysis of fish waste is to maximize the recovery of available recyclable components with high quality (degree of hydrolysis and high protein content) in them (19). Hydrolyzed proteins have a significant ability to inhibit the ability of hydroxyl radicals. Various methods are used to produce hydrolyzed proteins, including: enzymatic hydrolysis, autolysis, thermal hydrolysis and bacterial fermentation. In enzymatic hydrolysis method proteins are broken down into soluble and insoluble components by using specific enzymes. In autolysis method large molecules of proteins are broken down into small peptides by using the internal enzymes of fish. The value of proteins as the main source of amino acids in human health has been proven. In addition to nutritional value, proteins have biological functions. These proteins show their functions through bioactive peptides. Production methods of bioactive peptides affect their biological activity (20,21).

4- Decomposition and hydrolysis of marine proteins

In recent years, a significant number of studies have focused on the isolation of bioactive peptides in dietary proteins. These peptides act as functional nutrient compounds which promote heath level, they are healthy and are potential drugs for the treatment of chronic diseases. In essential protein, peptides are inactive and therefore must be free to exert an effect. These bioactive peptides usually contain 2-20 amino acids during their synthesis. Nevertheless some peptides have been reported to have more than 20 amino acids in their length. It is a method for obtaining peptides from protein food sources with different biological activities such as antioxidant, antihypertensive, antimicrobial and antiproliferative activity (20).

The type, number, sequence and properties of amino acids in bioactive peptides structure lead to the formation of different physiological activities. The

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bioactive properties of peptides depend on the number, type and sequence of amino acids in the protein polypeptide chain and the placement of nitrogen and carbon at the end of the chain. Bioactive peptides have valuable nutritional properties and improve the health of the body's organs. These compounds can be widely used in the production of functional foods and many medicines. Also, due to the fact that small peptide particles have less allergenicity than proteins, they can be used in infant formula. Today, about 152 types of peptides are used in the pharmaceutical and medical industries (22).

4-1 bioactive peptide production methods

Three methods include solvent extraction, enzymatic hydrolysis and microbial fermentation of marine proteins are used to produce marine bioactive peptides. Enzymatic hydrolysis methods are especially preferred in the food and pharmaceutical industries due to the lack of residues of organic solvents or toxic chemicals (21).

4-1-1 Enzymatic hydrolysis

The most commonly used protein hydrolysis method is enzymatic hydrolysis. Alkaline hydrolysis causes racemization and degradation of certain amino acids at high pH. On the other hand, the disadvantage of acid hydrolysis is that tryptophan may be completely destroyed and asparagine and glutamine may be hydrolyzed to their corresponding amino acids. Therefore, enzymatic hydrolysis is performed under controlled pH and temperature conditions to produce undesirable products. The enzymes used to obtain hydrolyzed products, include gastrointestinal and microbial proteases including alkalase, trypsin, pepsin, and chymotrypsin (23). Also, many researches have been done on enzymatic hydrolysis of different parts of fish using commercial enzymes. Many of these studies have examined the effect of a variety of enzymes, including plant-derived papain enzymes, alkalase, protamex, fluorosyme, neotraz and promod, originating in Microbial. chemotrypsin and trypsin are animal origin (24). In general, enzymes of microbial origin have more advantages than other enzymes, including greater stability to temperature, high pH and suitable proteolytic properties (25). In addition, studies have shown that enzymatic hydrolysis is likely to increase the antioxidant activity of hydrolysates by increasing the activity of free radical scavenging (18,26). Enzymatic hydrolysis of dietary proteins is an efficient method for retrieving potentially biologically active peptides. There is found in hydrolyzed products of marine proteins (27). This process protects functional properties of proteins such as solubility, emulsifying capacity and water holding capacity. It also improves gel formation. In addition, it has been reported that the allergenic properties of some proteins, such as beta-globulin and ovalbumin, are reduced by enzymatic hydrolysis (28). Bioactive peptides such as fish bones, shrimp appendages and lesions have been identified after enzymatic hydrolysis of various marine wastes (27,29).

4-1-2 Microbial fermentation

Fermentation is one of the oldest methods used to preserve food. In East Asian countries, such as China, Japan, and Korea, fermented fish products, crustaceans, and mollusks are used as staples or seasonings. Fermented products contain amino acids and peptides that give the product a special flavor and aroma. During the fermentation process, the breakdown of proteins by microbes and their

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proteolytic enzymes increases the nutritional-medicinal value and shelf life of the product (30, 31). During the fermentation process, sugars and proteins are broken down by microorganisms and peptides will be formed with new amino acid sequences. Many bioactive peptides can be produced by proteolytic microbes in fermented dairy products. Microbial fermentation is a less expensive method of producing bioactive peptides than enzymatic hydrolysis. Because microorganisms are a cheaper source of protease enzymes. The cost of bacterial cultures is also low due to low nutritional needs and short growth time. Also, proteases of lactic acid bacteria are located on the cell wall, which makes extraction protocols easier and cheaper (22). There are also methods in which it is possible to produce bioactive peptides by combining both enzymatic hydrolysis and microbial fermentation methods (17).

5- Effective factors on antioxidant mechanism of bioactive peptide

Peptide size, type of amino acids, location amino acids in different chain positions and hydrophobicity are the most important factors that affect the mechanism antioxidant peptides. Antioxidant peptides can be used as beneficial compounds for prevent the oxidation of fats and proteins used in food. These peptides are involved in oxidative processes and have potential importance in the treatment and control of diseases (32). Some factors such as protein substrate type, raw material treatment, type and concentration of enzyme, temperature, reaction time and protein density affect peptide structure and thus antioxidant function (29,33).

5-1 Antioxidant mechanism of bioactive peptides

Bioactive peptides have strong antioxidant activity against free radicals and other reactive oxygen species (ROS). The mechanism by which peptides exert their antioxidant effects has not been fully elucidated, although various studies have shown that hydrolyzed peptides and proteins inhibit enzymatic and non-enzymatic oxidation by removing free radicals and chelating metal ions (32,34). These peptides inhibit oxidation through various mechanisms, including inhibition of fat oxidation, removal of free radicals and chelation of metal ions. Inhibition of oxidation improves the function and health of cells and various organs of the body and also prevents food oxidation spoilage. Glutathione-like antioxidant peptides (γ-Glu-Cys-Gly) (β-alanyl-L-histidine carnosine which are naturally present in muscle tissue, prevent the lipid oxidation by giving electrons and chelating metal ions (27).

The effect of molecular weight of peptides on their antioxidant activity has been emphasized in a number of studies (35). However some others point to the effect of the amino acid combination of peptide and hydrolyzed protein on their antioxidant properties (27,36). Enzymatically hydrolyzed fish skin gelatin shows better bioavailability than peptides derived from fish muscle protein. It has antioxidant and antihypertensive properties (35). Gelatin peptides have unique replicated glycine-proline-alanine sequences in their structure and it is thought that the antioxidant and antihypertensive properties of gelatin peptides may be related to their unique amino acid composition (37).

6- Extraction and purification of bioactive peptides

The product of enzymatic hydrolysis of proteins contains a variety of peptides with different lengths and amino acid compositions. Therefore, isolation and purification of bioactive peptides is an important part of the process of identifying bioactive peptides and determining their physical and chemical

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properties and evaluating their biological activity (38). Bioactive peptides can be separated from protein hydrolysis products by various methods including membrane separation techniques and chromatography (39). In membrane separation method, the protein hydrolysis product is poured using pressure on the surface of the porous membrane and depending on the membrane characteristics, the separation is done based on peptide size or electric charge. Membranes are divided into microfiltration, ultrafiltration (UF) and nanofiltration (40). Chromatography is mostly used in combination with quantitative and qualitative analyzing equipment such as UV detectors or mass spectrometers. Usually a combination of several techniques is used to isolate and purify peptides. For example, first the peptide components are separated using membrane filtration and then purification is performed using reverse phase chromatography (RPC). Purified peptides can be subjected to qualitative analysis protein sequencer and amino acid analyzer is the most common devices used to determine amino acid composition and sequence. Other methods for identifying peptides include detection, fluorescence detection, mass spectrometry, electrospray ionization – tandem mass spectrometry (41). Many studies have reported the biological activities of tilapia protein hydrolyses, including antioxidant (42), angiotensin converting enzyme (ACE)-inhibitory (43) and calcium-binding (14) activities. The main challenge associated with the generation of bioactive peptides from tilapia is the decrease in stability of protein hydrolyses and in bioactivity due to the presence of prooxidants such as heme and lipids (43). These unstable materials can be reduced through different protein isolation methods including acid or alkaline treatment (44). A developed alkaline extraction is followed by the isoelectric precipitation approach for the isolation of myofibrillar proteins with low lipid content by adjusting to high pH with alkaline solution and then precipitating proteins at pH 5.5 with acid solution. In the extraction methods of collagen or gelatin, alkaline pretreatment has been widely applied to remove pigments, lipids and non-collagen proteins from fish skin (45). Table 2. shows the extraction and purification of bioactive peptides (40).

Table2. Extraction and purification of bioactive peptides (40).

Disadvantages	Advantages	Technique
No inhibition of polar molecules, Slow diffusion of samples between particles, Secondary matter interactions solve matter with stationary phase	Useful for separating complex mixtures of peptides	Reversed-phase high-performance liquid chromatography (RP-HPLC)
Low selective separation, required additional steps for separation	Isolation of peptide mixtures which contains anionic and cationic peptides	Ion exchange chromatography (IEC)
Bandwidth widening and reduced performance due to the heat generated due to the use of very fine particles and high pressure	Increased sensitivity, clarity in separation of the mixture A complex peptide	High performanc liquid chromatography (UHPLC)
Requires a long column for isolation of peptide compound complicated	Minimal effect on the structure and properties of proteins	Size exclusion chromatography

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In the membrane separation method, the product of protein hydrolysis is poured on to the surface of a porous membrane by using pressure. Depending on the characteristics of the membrane, the separation will done based on the size of the peptides or their electrical charge. This method uses a variety of microfiltration, UF and nanofiltration membranes. RPC is one of the common methods of separation and purification of peptides that allows rapid separation and identification.

It also determines the hydrophilic and hydrophobic properties of peptides. Chromatography is mainly used in combination with quantitative and qualitative analysis equipment such as UV or mass spectrometry. Separation and purification of peptides is usually done using a combination of several techniques.

For example, peptide components are first separated using membrane filtration and then purification process is performed using RPF. Finally, the purified peptides are qualitatively identified. The most common methods used for the separation and enrichment of peptides include UF, ion exchange, gel filtration, and liquid chromatography. Although most of these methods are effective on a laboratory scale, they are not suitable for large-scale due to the high cost of production (46).

After achieving the desired degree of hydrolysis, the obtained product are performed through filtration or oven chromatography techniques in order to fractionation and partial purification. The most common methods used for peptide purification are UF, RPC, IEC, gel filtration chromatography (GFC) and high performance chromatography (HPLC). RPC is also the most widely used for classification of peptides and their metabolites (47).

Conclusion

This review article due to the importance of fisheries in human health and the large amount of waste during its processing is provided. Tilapia has been welcomed and has paid more attention in many countries around the world because of it is fast breeding and marketability. Hydrolyzed fish proteins have a wide range of applications and the value of proteins as a major source of amino acids in human health has been proven.

The product of enzymatic hydrolysis of proteins contains a variety of peptides with different lengths and amino acid compounds. Isolation and purification of bioactive peptides is an important part of the process of identifying bioactive peptides. Fish waste products is a potential source of bioactive compounds which can be obtained by different methods including enzymatic hydrolysis, fermentation and solvent extraction.

The results of this study support the idea that the enzymatic hydrolysis method is a perfect technique for the extraction of bioactive peptides. It is also more stable and safer than solvent fermentation and extraction methods. In order to isolate, purify and identify peptides with the highest antioxidant properties ultra membranes, filtering, chromatography and mass spectrometry are used. Biopeptides have significant physiological properties such as reducing the risk of chronic diseases, increasing the function of the immune system, antimicrobial activity, anti-oxidant properties, anti-clotting, anti-hypertensive, anti-cancer and cholesterol lowering.

According to the results of this research, aquatic wastes is a good source of biopeptides and could be employed as a raw material for the producing functional foods and improve the community health. Therefore, the

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remaining fish wastes are considered as a valuable resource, it can also lead the reduction of the contamination that might been produced by these wastes. It is hoped that the experts and investors use the aquatic wastes properly to produce high value-added materials.

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استخراج و خالص‌سازی پپتیدهای زیست فعال از ضایعات ماهی تیلاپیا (*Oreochromis*)

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صفحه ۶۰-۴۹

چکیده

در صنایع شیلات در مقایسه با سایر صنایع غذایی، حجم بالایی از محصولات جانبی مانند استخوان، اضافات گوشت، پوست و امعاء و احشاء تولید می‌شود که تیمار کردن و دفع آنها در محیط زیست هزینه‌ی هنگفتی در پی دارد. ماهی تیلاپیا با توجه به تکثیر سریع، بازارپسندی و حجم بالای ضایعات مورد توجه قرار گرفته است. ارزش پروتئین‌ها به‌عنوان منبع اصلی آمینواسیدها در سلامت انسان اثبات شده است. پروتئین‌ها علاوه‌بر ارزش غذایی دارای عملکردهای زیستی هستند. این پروتئین‌ها عملکردهای خود را به‌واسطه پپتیدهای زیست‌فعال نشان می‌دهند. روش های تولید پپتیدهای زیست‌فعال بر فعالیت زیستی آنها مؤثر است. استفاده از روش هیدرولیز موجب پایداری آنها نسبت به روش‌های تخمیری و استخراج با حلّال می‌شود و روش ایمن‌تری نسبت به دو روش دیگر است. پپتیدهای زیست‌فعال دارای تأثیرات فیزیولوژیکی نظیر ضد فشار خون بالا، آنتی‌اکسیدانی، ضدمیکروبی، ضدسرطانی و ضددیابتی بوده و دارای کاربردهای بسیاری در صنایع غذایی، دارویی و آرایشی هستند و از آن‌ها می‌توان در صنعت غذا به‌عنوان مواد غذایی فراسودمند استفاده کرد.

واژه‌های کلیدی: آبزیان، بیوتکنولوژی، پپتیدهای زیست‌فعال، تیلاپیا، ضایعات.

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