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Biologically active peptides derived from egg proteins

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During food processing, proteins are subjected to different modifications, of which most are the result of proteolytic enzyme activity. Due to these mild and easily controlled reaction conditions, this method of modification has been commonly applied in producing high quality food products. Processing affects the conformation of the molecule and its biological activity. Limited hydrolysis leads to improvement of both the functional and nutritional properties of proteins, as well as their organoleptic characteristics. Proteolytic enzymes release peptides that frequently show different specific biological activities. Bioactive peptides govern the function of other compounds in the bloodstream, digestive, immune and nervous systems. Food sources of bioactive peptides exhibiting anti-hypertensive, anti-tumour, antioxidant or antimicrobial activities include milk, eggs and fish. Biopeptides with specific biological activities can be used in pharmaceutical, cosmetic or food industries as natural substances which prevent adverse changes, as food additives or as functional food ingredients with defined benefits to the consumer. The following paper reviews the role of hens eggs as a source of bioactive peptides, and how these can be used in functional foods.

Keywords: bioactive peptides; hydrolysis; eggs; antioxidant; immunostimulation

Introduction

Dietary protein hydrolysates are mixtures of peptides of varying molecular weights and free amino acids, which show a higher absorption rate compared to native proteins. They are widely used in the manufacturing of dietary supplements and food for particular nutritional uses (Clemente, 2000). Enzymatic hydrolysis of protein is mainly carried out under industrial conditions. Processes catalysed by enzymes are highly specific, and their progress can be fully controlled. Protein hydrolysates with a certain degree of hydrolysis

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are a valuable source of peptides that are characterised by high biological activity and nutritional value (Clemente, 2000; Hartman and Miesel, 2007).

Diet has a vital role in shaping the health of the body. Bioactive peptides are good examples of substances that exert different health effects. They are released from food proteins by the action of proteolytic enzymes (Hartman and Miesel, 2007). Distinguished opioid peptides have been identified as compounds with immunostimulating, blood pressure lowering, platelet aggregation inhibiting, antimicrobial, antioxidation and mineral absorption-enhancing properties (Mine and Kovacs-Nolan, 2006; Hartman and Miesel, 2007). The specific activity of peptides is determined by the type and location of amino acid residues contained in their primary structures (Park *et al.*, 2001). Proteins from food material are the precursors of biopeptides. This protein may be either of animal or plant origin such as milk proteins (casein, whey protein), muscle proteins (myosin, collagen), soy protein (glycinin, β-conglycinin) wheat (gluten) protein or egg (ovalbumin, conalbumin) (Tsuge, 1991; Ibrahim *et al.*, 1998; Pellegrini *et al.*, 2000; Saiga *et al.*, 2003; Gibbs *et al.*, 2004; ; Kim *et al.*, 2007; Kong *et al.*, 2007; Li *et al.*, 2007).

Enzymatic hydrolysis of proteins

Hydrolysis of proteins is a process of decomposition into individual amino acids and/or peptides that occurs with the participation of enzymes. The rate of hydrolysis depends on many factors such as enzyme specificity and concentration, substrate concentration, the rate of mass transfer in the enzyme/substrate system, the degree of denaturing of the protein in the substrate, pH, temperature, ionic strength in the reaction medium and the presence of activators and inhibitors (Kołakowski *et al.*, 2005). The hydrolysis process can proceed according to two mechanisms, classified as 'one-by-one' or 'zipper'. During the initial stage of the reaction type 'one-by-one' (determining the overall level of hydrolysis) it is necessary to partially unfold native protein molecules. As a result, the protein loses its stability, more peptide bonds are exposed on the outside of the molecule (intermediate products) and the enzyme has access to the hydrolyzed peptide bond. In a further step, intermediate products are very rapidly degraded to small peptides. If the reaction equilibrium is on the 'native side', then the protein molecules are degraded one by one, so there are no significant quantities of intermediate products; only native proteins and final products are present. In the 'zipper' type reaction, native protein is very rapidly converted to intermediate products, and hydrolysis of indirect forms of protein in the final products occurs very slowly (Kunst, 2003).

Proteolysis may occur by the one-or two-step system. In the case of one-step hydrolysis, the pH of the mixture is changed automatically as a result of the reaction, or is maintained at a constant level by the addition of acids or bases. That hydrolysis takes place in a continuous manner. However, during the two-stage hydrolysis, the pH of the reaction varies in subsequent stages of proteolysis through the use of the same enzyme or using a variety of enzymes, *e.g.* firstly endopeptidase and then exopeptidase (Kołakowski *et al.*, 2005).

The hydrolysis of proteins significantly modifies the functional and sensory properties of the product. This is due to changes in the third-row structure of proteins and molecular weight reduction (Vinnars and Wilmore, 2003). Most enzymatic modifications of proteins are carried out with the participation of enzymes such as pepsin, bromelain, trypsin, chymotrypsin, papain or ficin. Protein degradation and increasing the degree of proteolysis (% DH) significantly improves frothiness, solubility, emulsifying capacity and gelling (Vinnars and Wilmore, 2003; Agyare *et al.*, 2008). For example, selective enzymatic hydrolysis under controlled conditions is used to improve the solubility,

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emulsifying and foaming properties of wheat gluten (Agyare *et al.*, 2008) and fish protein (Shahidi *et at*., 1995). Rapeseed protein hydrolysates have much better emulsifying properties and water-binding capacity than the starting substrate. While the use of sequentially alkalase and exopeptidase allows for highly soluble hydrolysates, which can be used as components of specialized high-protein liquids (Vioque *et al.*, 1999).

Non-specific, far-reaching hydrolysis may lead to loss of functional properties and the appearance of bitter peptides. The bitter taste of protein hydrolysates can be reduced to an acceptable level by means of ultra filtration, activated carbon processing and utilising the plastein reaction (Clemente, 2000). It has been proven that, by using certain enzymes in the hydrolysis process, the bitter peptides can be significantly masked (FitzGerald and O'Cuinn, 2000). The development of biotechnological solutions to obtain hydrolysates without a bitter aftertaste is already in progress.

Protein hydrolysates are produced on a large scale from various sources such as milk, eggs, fish, rice, legumes, meat, and potatoes (Schaafsma, 2009). Their valuable properties make them excellent ingredients for increasing the share of nitrogen in specialty drinks, manufacturing specialist foods for pregnant women, nutritional and cosmetic ingredients and in dietary supplements. They can also be found in foods for people with impaired digestion, for athletes and in hypoallergenic products (Schaafsma, 2009). In addition, hydrolysates are useful as a growth media for a variety of desirable microorganisms. Several studies have confirmed the positive impact of the fraction of peptides added to media in order to improve the growth of microorganisms or in the production of recombinant proteins (Duarte de Holanda and Netto, 2006).

These days hydrolysates derived from marine products have become particularly popular, mainly due to the fact that they contain a high amount of complete amino acids (Córdova-Murueta *et al.*, 2007). Over the years, techniques have been developed in the use and recovery of valuable by-products in biopolymers (Ferrer *et al.*, 1996).

Bioactive peptides - sources and application

The presence of biopeptides in hydrolysates has aroused great interest amongst scientists (Park *et al.*, 2001; Korhonen and Pihlanto, 2003; Hartman and Miesel, 2007). Bioactive peptides are fragments of proteins that are not active in the sequence of the precursors. After enzymatic release they interact with the corresponding receptors to regulate the body and its functions (Kołakowski *et al.*, 2005). They cause various health effects, acting as human hormones on the circulatory, digestive, nervous, and immune systems (Hartman and Miesel, 2007) (*Table 1*). The specific activity of a peptide is determined by the type and location of amino acid residues contained in their primary structure (Park *et al.*, 2001). Among the bioactive peptides, simple and complex dipeptides, linear or cyclic oligo-and polypeptides exist which may exhibit various activities (Mine and Kovacs Nolan, 2006; Hartman and Miesel, 2007). Moreover, some bioactive peptides exhibit chemotactic properties, embryotoxic properties, hemolytic properties, inhibitory activity, cause smooth muscle contractions by stimulating the synthesis of red blood cells and regulate the activity of the stomach mucosa and the flow of ions (Kołakowski *et al.*, 2005). Typically, peptides have multiple biological activities (Korhonen and Pihlanto, 2003).

Antioxidant activity

Oxidative reactions underlie the adverse changes in food products (rancidity, odour and colour change) and most pathological conditions (cardiovascular diseases, degenerative

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changes) (Saiga *et al.*, 2003). Currently, great attention is paid to compounds of natural origin, *e.g.* in the potential therapeutic role of antioxidants, which are offset by unfavourable changes resulting from the action of oxygen free radicals. Natural sources of powerful antioxidants such as protein hydrolysates are considered useful in preventing disease caused by oxidative stress, such as colon cancer, Alzheimer's disease and Parkinson's disease (Park *et al.*, 2001).

Table 1 Examples of bioactive peptides derived from food.

Effect	Source of peptides	Encrypting protein(s)	Name/remarks/sequence (in single-letter code)	Reference
Antioxidant	egg	lecithin-free egg yolk	LMSYMWSTSM LELHKLRSSHWFSRR	Park *et al.* (2001)
	egg	Ovalbumin	AHK, VHH, VHHANEN	Tsuge *et al.* (1991)
	milk	α-lactalbumin β-lactoglobulin	MHIRL, YVEEL, WYSLAMAASDI	Hernandez-Ledesma *et al.* (2005)
	wheat	germ protein	peptides not specified	Zhu *et al.* (2006)
ACE inhibitory/ Hypotensive	egg	ovotransferin ovalbumin	KVREGTTY ovokinin (FRADHPPL) ovokinin (f 2–7) (KVREGTTY)	Lee *et al.* (2006) Miguel and Aleixandre (2006)
	milk	α-lactalbumin β-lactoglobulin, α^{-}, β^{-}, κ-casein	lactokinins (*e.g.* WLAHK, LRP, LKP) casokinins	Mizushima *et al.* (2004)
	fish	miofibryllar protein hydrolysates	(*e.g.* FFVAP, FALPQY, VPP) oligopeptide LKPNiofibryllar protein hydrolysates	Hartman and Miesel (2007)
Antitumour	egg	Phosvitin	phosphooligopeptides	Picot *et al.* (2006)
	fish	miofibrillar protein hydrolysates	peptides not specified	Mine and Kovacs-Nolan (2006) Pellegrini *et al.* (2004)
Antimicrobial	egg	ovotransferrin lysozyme ovalbumin	OTAP-92 (f 109–200) peptides not specified penta-, hexa- and oktapeptides	Choi *et al.*, 2005
Metal binding	egg	Phosvitin	phosphooligopeptides	Cross *et al.* (2007) Miquel and Farre (2007)
	milk	α-, β- casein	caseinophosphopeptides	Gauthier *et al.* (2006)
Immuno-modulatory	milk	α-, β-, κ- casein, α-lactalbumin	immunopeptides (*e.g.* aS1-immunocasokinin) (TTMPLW)	Mine and Kovacs-Nolan (2006)
	egg	Ovalbumin	peptides not specified	Iwaniak and Minkiewicz (2007) Qian *et al.* (1995)
Antithrombotic	milk	κ-casein	caseinoglycopeptide (CGP) (f 106-171)	Chavakis *et al.* (2002)
	pork	miofibryllar protein hydrolysates	FK633, His-Gly-Lys	

The antioxidant activity of protein hydrolysates depends largely on the degree of hydrolysis (% DH) and the type of proteases used for enzymatic degradation (Kong and Xiong, 2006). The crucial factor is the weight, type and position of amino acid

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residues contained in the structure of the peptides (Park *et al.*, 2001). Marcuse (1962) demonstrated that antioxidant properties are determined by the presence in hydrolysates of several amino acids, such as tryptophan, methionine, histidine, lysine, glycine. Kawashima *et al.* (1979) found that the aromatic amino acids, present in some di-and tri-peptides, exhibit strong free radical scavenging properties. This is due to the ability of these amino acids (tyrosine, tryptophan and histidine) to readily cleave hydrogen - an electron donor to free radicals seeking to adopt a stable form through their pairing (Park *et al.*, 2001; Davalõs *et al.*, 2004). The mechanism of antioxidative hydrolysates and peptides may be manifested as the scavenging of free radicals, chelation of transition metals, reduction of hydroperoxides, enzymatic elimination of specific oxidants or inhibition of lipid peroxidation in food systems (Elias *et al.*, 2008).

Many antioxidant peptides have been prepared from wheat (Liyana-Pathirana and Shahidi, 2007), maize (Kong and Xiong, 2006), pig blood haemoglobin (Chang *et al.*, 2007), or eggs. For example, two peptides with aromatic residues, which were obtained from egg yolk proteins under the influence of Alcalase have the potential to inhibit the oxidation of linoleic acid (Park *et al.*, 2001). Ovalbumin is the source of three antioxidant peptides containing sequences in the second position the rest of the histidine (Tsuge *et al.*, 1991). A characteristic feature of these peptides is the presence of the rest of the leucine at position N-terminal, conditioning their bioactivity (Park *et al.*, 2001). A similar effect has antioxidant preparation obtained by the action of bacterial proteolytic enzymes (Bacillus sp) on the yolk protein, consisting of a fraction of peptides with molecular weights not exceeding 1 kDa (Sakanaka *et al.*, 2004).

Further peptides with antioxidant activity are products resulting from the hydrolysis of ovalbumin with pepsin, which have the ability to scavenge free radicals AAPH (Davalõs *et al.*, 2004). Many valuable antioxidant peptides were also obtained from phosvitin - a highly phosphorylated glycoprotein, represents the major fraction of egg yolk phosphoproteins. Peptides derived from phosvitin are characterised by a high content of phosphorus and amino acids such as histidine, methionine and tyrosine (Xu *et al.*, 2007). It's also been shown that phosvitin products, by inhibiting iron-dependent ion formation reaction of the most reactive hydroxyl free radicals, are linked to DNA protective properties (Matoba *et al.*, 1999).

Studies in recent years confirm that some protein hydrolysates and peptides exhibit better antioxidant activity than α-tocopherol and may even exceed the activity of potent synthetic antioxidants (BHA, BHT) used in the food industry (Je *et al.*, 2007). In addition, many antioxidant peptides have a multifunctional character, showing other properties such as antihypertensive, opioid, anti-thrombotic and hypocholesterolemic which makes these compounds more useful as dietary ingredients in promoting human health (Davalõs *et al.*, 2004).

Anti-hypertensive activity

Among the most increasing lifestyle diseases, including those stemming from an unhealthy lifestyle, cardiovascular disease and hypertension are of key importance. Peptides can be considered natural antihypertensive pharmacological compounds. They inhibit the activity of angiotensinogen I-converting enzyme (ACE), a peptidyl-dipeptidase that can cleave a dipeptide from the angiotensin I (decapeptide: DRVYIHPFHL) C-terminal, which causes its conversion to angiotensin II, a blood pressure booster. Inactivation of the enzyme by inhibitors means bradykinin stimulation also, which lowers blood pressure (Miguel *et al.*, 2006). These

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antihypertensive precursors of peptides are prominently found in egg albumin (Miguel and Aleixandre, 2006; Hartman and Miesel, 2007).

Great interest in biologically active peptides has been generated from work investigating ovalbumin – *i.e.* the proteins present in egg white. When the whole egg is eaten, ovalbumin is transformed into octapeptide under the influence of pepsin called ovokinin, representing fragment (f 358-365) of ovalbumin (Fujita *et al.*, 1995; Davis and Reeves, 2002). In addition, under the influence of chymotrypsin, ovokinin (2-7) is released (Matoba, 1999). These peptides act by lowering systolic blood pressure in rats, in a dose-dependent manner (Fujita *et al.*, 1995). In the digestive system, these peptides undergo immediate emulsification and are well absorbed by the intestine (Davis and Reeves, 2002).

Anti-tumour activity

Evidence of the benefits of consuming bioactive peptides has been recognized in the case of neoplastic changes (Erdmann *et al.*, 2008). Epidemiological studies show that the incidence of tumours in different parts of the human digestive system is strongly conditioned by the diet (Rose *et al.*, 2007). Studies show that the risk of colorectal cancer may be reduced under the influence of hydrolysates from egg yolk protein (Azuma *et al.*, 2000; Belobrajdic *et al.*, 2003), whereby ingestion inhibits the proliferation of tumour cells in the colon of rats. Specific protective effects against the proliferation of cancer cells have been attributed to phosvitin, as this protein and its peptides, have strong antioxidant properties *in vitro* (Ishikawa *et al.*, 2004) and in human intestinal epithelium (Katayama *et al.*, 2006). In addition, they regulate the level of the enzymes responsible for biosynthesis of glutathione (GSH), which plays a key role in the mechanism of cell death (Richman and Meister, 1975). Studies have shown that oligopeptides released from phosvitin have the ability to increase intracellular GSH activity and regulate the expression of γ-glutamycysteine in intestinal epithelial cells, which catalyses the synthesis of GSH (Katayama *et al.*, 2006; 2007). Phospho-oligopeptides obtained from phosvitin also have the ability to regulate the levels of antioxidant enzymes such as catalase and glutathione reductase. For this reason they can be considered as anticancer agents in the colon.

The precursors of anti-cancer peptides are present in the egg ovomucin. The action of pronase enzymes from *Streptomyces griseus* on both factions β and α of the proteins, released highly glycosylated oligopeptides with masses of 220 (f β), 120 (f β) and 70 (f α) kDa. Direct treatment of the mice tumours with these oligopeptides effectively inhibited tumour growth (Watanabe *et al.*, 1998).

Antimicrobial activity

Peptides derived from food protein hydrolysates can exhibit antimicrobial activity, especially those containing tryptophan and arginine. The presence of arginine in the peptide sequence allows the molecule to give a positive charge, which forms a hydrogen bond with the anionic components of the cell membrane, impacting on the peptide - cytoplasmic membrane (Powers and Hancock, 2003). However, the presence of tryptophan promotes the folding of peptides in aqueous solutions because it has an affinity to the cell membrane. This provides the structure of the amphiphilic peptides necessary for their transport across the cytoplasmic membrane (Chan *et al.*, 2006).

There are several mechanisms of action of peptides against microbial cells. One of

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them, which is used particularly in relation to gram-negative bacteria, is due to the ability of peptides to bind to the cell lipopolysaccharide wall. As a result of aggregation of peptides in the cytoplasmic membrane, channels are formed that cause destabilisation, leading to cell death. An example of a peptide showing this activity is lactoferricin B.

Peptide precursors with antimicrobial properties include ovalbumin, the major egg protein. Bovine trypsin is used to release of this protein as well as penta-, hexa-and oktapeptides which exhibit strong bactericidal activity against *Bacillus subtilis*. In contrast, peptides formed by the action of bovine chymotrypsin demonstrate bactericidal activity against Gram-positive bacteria, such as *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus epidermidis*, *Streptococcus zooepidermicus* and the Gram-negative bacteria *Bordetella bronchiseptica*, *Escherichia coli*, *Klebsiella pneumoniale*, *Pseudomonas aeruginosa* and *Serratia marcesens*, as well as fungi, such as *Candida albicans* (Pellegrini *et al.*, 2004). The antimicrobial activity shows that the 9.9 kDa fragment (f 109 -200) of conalbumin (OTAP 92) is active against G+ and G- bacteria. The OTAP peptide 92 obtained from the N-terminal fragment of the polypeptide chain and has a cationic character. The same features have been observed in peptides released by pepsin, of lactoferrin in human milk and cow's milk, or peptides formed during hydrolysis of basic pancreatic trypsin inhibitor by clostripain, (Ibrahim *et al.*, 2000). Moreover, the OTAP-92 peptide shows anti-virulence properties, which is effective in the inactivation of Marek's disease virus in chickens.

Lysozyme is another precursor of antimicrobial peptides. Partial hydrolysis of this protein using pepsin gives rise to a mixture of peptides exhibiting activity against bacteria of the genus *Bacillus*. These peptides retain activity even after 30 minutes heating at 95°C in a pH range from 4.5 to 7, making them useful a natural preservatives in processed food (Abdou *et al.*, 2007).

Metal binding peptides

Of great importance, especially in the food and pharmaceutical industries, are the metal ion binding peptides. They can serve as carriers of micronutrients, facilitating their absorption, due to the specific transport systems for short peptides in the digestive system (Clemente, 2000). Peptides with chelating ability may therefore be useful for increasing the bioavailability of minerals and to preventing the oxidation of food products, whilst retaining the visual character and quality of food. Phosphopeptides, derived from milk proteins, have chelating properties for metals such as calcium (Cross *et al.*, 2007), zinc (Miquel and Farre, 2007) and iron (Bouhallab *et al.*, 2002). Similar effects are seen with peptides derived from egg yolk phosvitin, which may increase the bioavailability of calcium (Choi *et al.*, 2005). Phosvitin contains more serine residues than casein. In addition, the high phosphorus content gives this protein some specific biological properties. It has been proven that the peptides from phosvitin increase calcium absorption in rats (Choi *et al.*, 2005). Other studies indicate that when these peptides are obtained by hydrolysis with trypsin, it increases the binding of calcium in the bones and prevens the formation of insoluble calcium phosphate (Jiang and Mine, 2001). As a result, phosphopeptides, derived from phosvitin, contain a large amount of phosphoserine and can be widely used as nutritional supplements and pharmacological preparations for people suffering from osteoporosis. Chelating peptides, by protecting the body against free radicals, may also improve the performance of physical and mental activity and delay the aging process in humans (Hartman and Miesel, 2007).

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Immunostimulating activity

The immune system has a decisive influence on the equilibrium state of health in the body which determines its homeostasis. One possible strategy to increase natural immune protection is the use of a balanced diet rich in probiotics and functional foods (Nakano *et al.*, 2001). In recent years the role of reducing the risk of chronic diseases and enhacing immune protection has been attributed to bioactive peptides derived from food proteins (Ma *et al.*, 2006).

The mechanism of action of biopeptides may rely on direct interaction with pathogens as well as suppression or stimulation of certain immune responses (Hancock and Sahl, 2006). Studies show that both immunosuppression and immunostimulation may be important in preventing and fighting various pathological conditions of the body, and peptides have an immunomodulating effect that can be used to reduce inflammation in autoimmune diseases, prevent transplant rejection and improve overall health (Gauthier *et al.*, 2006). There are numerous examples in the literature of such interactions (Biziulevičius *et al.*, 2006), however most are derived from milk.

Prevention of diseases associated with the current way of life, such as atherosclerosis, myocardial infarction or stroke is very important and necessary in many developed countries. The use of anti-peptide seems to be a promising strategy for reducing the risk of thrombotic disease in healthy people and assist or even replace the currently used therapeutic agents.

Conclusions

There is a growing interest in the food, pharmaceutical and cosmetic industries in the use of biopeptide preparations. Researchers are looking for unconventional, high-performance enzymes, and methods and technologies are being developed for specific hydrolytic processing. An example of this focus are the studies carried out in the project 'OVOCURA' within the consortium of the University of Life Sciences and Medical Academy in Wroclaw.

Understanding the relationship between physicochemical properties of protein hydrolysates and their functionality can lead to improved quality and stability of nutritional formulations based on the products of hydrolysis (Panyam and Kilara, 1996). Many potential hydrolysates require well-defined and repeatable enzymatic degradation processes. Because minor differences in the hydrolysis lead to changes in biological activity, the need for careful control of the process is paramount. Moreover, most of the identified physiological properties of bioactive peptides have been studied only *in vitro* or in animal models. Clinical trials on humans are limited or are not performed at all, because it is difficult to determine the level of bioactive peptides in serum. It should also be noted that, despite the great variety of bioactive peptides generated by the process of digestion in the human body, only the di- and tri-peptides are absorbed by the intestinal epithelial cells and transported by erythrocytes (Jones and Tung, 1983).

One of the problems concerning the efficiency of biopeptides is their oral application. To get into the bloodstream, they must overcome the body's natural barriers, such as a highly acidic stomach environment, pepsin and pancreatic enzymes and intestinal absorption. Therefore, it is necessary to conduct further research to identify the potential therapeutic role of bioactive peptides, derived from various sources, on the human body.

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