Studies in isolation and application of plant based bioactive peptides

Dissertation-I Report

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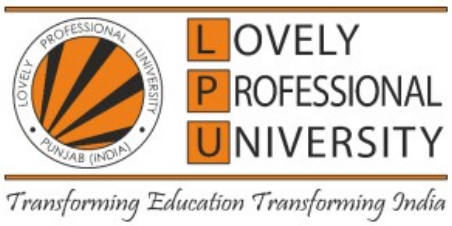

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CERTIFICATE

This is to certify that Divya Bhandari has personally completed M.Sc. Dissertation 1 entitled, "***Studies in isolation and application of plant based bioactive peptides***" under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation and study. No part of dissertation work has ever been submitted for any other purpose at any University.

The project report is appropriate for the submission and the partial fulfilment of the conditions for the evaluation leading to the award of Master of Food Technology.

Signature of Supervisor

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DECLARATION

I hereby declare that the work presented in the dissertation 1 report entitled "***Studies in isolation and application of plant based bioactive peptides***" is my own and original. The work has been carried out by me at the School of Agriculture, Lovely Professional University, Phagwara, Punjab, India under the guidance of **Dr. Yogesh Gat,** Assistant Professor (Food Technology) of the School of Agriculture, Lovely Professional University, Phagwara, Punjab, India, for the award of the degree of Master of Science in Food Technology.

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I certified that the above statement made by the student is correct to the best of my knowledge and belief.

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CONTENTS

Bioactive peptides are the proteins derived fragments which when consumed by humans provide positive influence on health by acting as the sources of proper nutrition and demonstrates numerous potential physiological functions in the body. In our body, these peptides are released as such by enzymatic proteolysis during gastrointestinal digestion. In vitro, peptides from foods can be released by hydrolysis of proteins with the use of food grade proteolytic enzymes and also during the processing of foods (cooking, ripening, fermentation), small fragments of bioactive peptides can be obtained in hydrolyzates (Abdel-Hamid et al., 2017). The bioavailability of isolated peptides majorly depends on the degree of hydrolysis during the isolation which is to be determined in vitro and in vivo for their commercialization.

There is a global prevalence of diseases like cardiovascular diseases, blood pressure, diabetes, cancer, etc. and direct treatment for these diseases has always been through a pharmaceutical approach which can lead to drug toxicity. Also, prolonged usage of any medication has its own negative side effects which can lead to deterioration in health, thus, increase in health care costs. Therefore, change in the era, demands the prevention through healthy lifestyle choices and intriguing in early treatments of individuals. Hence, natural food sources containing these bioactive peptides can significantly help in preventing the diseases and also, reducing the health care costs globally, decreasing the dependence on drug therapy.

Emerging new researches are focusing on the development of functional foods and Nutraceuticals, which involves the application of bioactive peptides which endeavor their respective physiological/health benefits. Hence, there is need to increase the commercial use of these peptides in the market by incorporating in various food products and making them bioavailable to claim the proper health functions in our body. In this review, we have discussed the isolation methods, their physiological functionalities and their bioavailability and safety concern taken into consideration.

CHAPTER 2 PROBLEM BACKGROUND

Humans have always been subjected to various health diseases due to changes in their physiological functioning on exposure to extrinsic environment. These changes can be controlled by homeostatic regulation or by the use of different health aiding factors for a specific disease (Ames and others, 1993). Nowadays, due to increase in awareness for the use of minimal drugs, the demand and market for functional foods and nutraceuticals is expanding. Hence, there is more interest developing on the studies related to bioactive peptides which can be used as a functional component in different food products to provide different health related benefits to the consumers preventing them from various acute or chronic diseases. Bioactive peptides are released by enzymatic hydrolysis of major food proteins followed by different separation techniques to isolate them from the mixture of other components, further incorporating into various products leading to more diversification and fulfilling different nutritional requirements, promoting health benefits (Wang and Gonzalez De Mejia, 2005; Korhonen and Pihlanto, 2006).

3.1. Defining bioactive peptides

Bioactive peptides are the derived fragments from the food protein which are the source of different amino acids and henceforth, adequate nutrition alongwith providing various health benefits by affecting physiological functions within the body (Harnedy & Fitzgerald, 2011; Sharma, Singh & Rana, 2011). Also, they find resemblance to hormones or drugs in their activity i.e. binding to target site and modulating physiological functions (Fitzgerald & Murray, 2006).

3.2. Sources of bioactive peptides

There are various plant and animal based sources for the isolation of bioactive peptides. Many studies have already been conducted with the use of different milk and milk products, plant based and different marine sources. As an example, casein phosphorylated peptides were the first peptide isolated from the milk in 1950. Food proteins are selected as a source of bioactive peptides on the basis of two criteria– 1) a pursuit of value-added use of abundant underutilized proteins or protein-rich food industry by-products, 2) utilization of proteins containing specific peptide sequences or amino acid residues of particular pharmacological interest (Udenigwe et al., 2012).

Marine source	Species	Bioactive peptide	Reference
Sponges	*Jaspis spp.*	Jaspamide	Odaka et al. 2000
	Heniastrella spp.		
	Auletta hemiasterlins	Hemiasterlins	Gamble et al. 1999
	Siphonochalina hemiasterlins		
	Geodia corticostylifera	Geodiamolide H	Frietas et al. 2008
	Dysidia arenaria	Arenastatin A	Kobayashi et al. 1994
	Homophymia sp.	Homophymines	Zampella et al. 2008
	Haliclona nigra	Haligramides A and B	Rashid et al. 2000
Mollusks	*Conus magus*	Ziconolide	Olivera et al. 2000
	Dolabella auricularia	Dolastatins	Bai et al. 1990
	Pleurobranchus forskalii	Keenamide A	Wesson et al. 1996
	Elysia rufescens	Kahalalides	Garcia rocha et al. 1996
Ascidians	*Tridemnum solidum*	Didemnin B	Palanisamy et al. 2015
	Styela sp.	Styelin D	Taylor et al. 2000
Seaweeds	*Eucheuma serra*	Lectins	Hori et al. 2007
	Porphyridium	Phycobiliproteins	Harnedy et al. 2011
	Spirulina		

3.3. Means of production of bioactive peptides

Bioactive peptides include the sequence of amino acids which are present in inactive form and can be produced from precursor proteins by following methods – 1) Enzymatic hydrolysis by digestive enzymes and 2) Fermentation of precursor proteins by proteolytic starter cultures

Studies have shown that combination of the above mentioned methods results in producing short chain functional peptides (Korhonen & Pihlanto, 2007). The importance of each of the methods is specific, as in, hydrolysis of peptides is important to liberate the potent peptides from the parent proteins because of their more bioavailability in target tissues. Also, the bioactivity of peptides majorly depend upon the enzymes used for hydrolysis, conditions of

processing and size of the isolated peptides. The size of the active peptide sequence varies in the range of 2 – 20 or even more amino acid sequences and they may possess multifunctional properties.

3.3.1. Enzymatic hydrolysis by digestive enzymes

The most typical method for the production of bioactive peptides from different food proteins is enzymatic hydrolysis. Different proteolytic enzymes are used such as papain, pepsin, protease, pancreatin, trypsin, chymotrypsin, alkalies, thermolysin, alongwith the enzymes from bacterial and fungal sources have been used to generate these peptides from different protein sources, depending upon the parent protein to produce protein hydrosylates. Specific conditions are to be set up to carry out the enzyme hydrolysis starting from pH conditions, enzyme to substrate ratio hydrolysis time and temperature at which reaction is to be carried out. This method is considered to be more suitable as compared to microbial fermentation as it is easy to adapt i.e. more feasible to conduct, short reaction time, and predictability.

A study describes that subtilisin enzyme produce smaller low molecular weight peptides, out of which, some are bioactive. Also, during the hydrolysis of snail foot muscle protein, i.e. *Achatina Fulica,* and rice bran proteins, subtilisin produced excessive number of smaller bioactive peptides when compared to papain, trypsin, cysteine endopeptidase and pepsin in their respective studies. Depending upon the type of enzyme used, the biological activity and the sequence of peptides varies. Proteases which result in low molecular weight peptides (<10kDa) are preferred as these are more effective antioxidants and antihypertensive peptides.

Following hydrolysis of proteins, mixture obtained is centrifuged and low molecular weight peptides need to be separated using various techniques like freeze drying desalting, gel filtration, cross-flow membrane filtration and column chromatography based on their sizes and are further being assayed.

3.3.2. Fermentation of precursor proteins by proteolytic starter cultures

Microbial fermentation is next feasible way for isolation of bioactive peptides. Many starter and non - starter cultures which we use in industries for production of fermented products are proteolytic in nature. Hence, those bacterial cultures are used for the isolation and release of these peptides from food protein sources. Bacteria or yeast cultures are grown over protein substrates which, when release enzymes result in hydrolysis of proteins and release of

peptides. The extent of hydrolysis mainly depends upon the type of starter culture used, a protein source and fermentation time. The studies showed that Lactobacillus brevis fermented whey exert a stronger ACE inhibitory effect than those incorporated with other *Lactobacillus spp*. Also, different ACE inhibitory and antioxidant activities were observed when milk was fermented using 14 different starter cultures. Hence, it was concluded that the functionality of hydrolyzed peptides depends upon the strain used for hydrolysis as each micro-organism has a unique proteolytic system. However, this method came out to be less useful for isolation of peptides from meat proteins. Also, no bioactive peptides have been produced by fermentation from muscle proteins. Isolated proteolytic enzymes from microorganisms can also be used to release bioactive peptides from proteinaceous raw material yielding different health benefits.

Further processing of peptides includes the use of different recent technologies such as sonication , hydrostatic pressure treatments, membrane ultrafilteration, size exclusion chromatography, electrodialysis ultrafilteration (EDUF), etc. depending upon the size, net charge and the functionality of the respective peptides to obtain pure peptides isolates to increase their potency.

3.4. Physiological functions of bioactive peptides

3.4.1. Antihypertensive

Antihypertensive peptides are the peptides which once released from the parent protein have the ability to modulate the renin – angiotensin systems and regulate blood pressure in the body. This hypertensive effect has been already put to use with the application of commercial drugs and now, this effect is also known to be shown by ACE inhibitory peptides which have been isolated from barley, garlic, sunflower, gelatin, etc. with the use of β – lactoglobulin, α – lactalbumin, γ – Zein, like enzymes.

A fraction of peptides of sequence SDNRNQGY, IQVPL and KGLWE isolated from egg yolk proteins exhibited 69.2% of ACE inhibitory activity which can be explained due to the presence of leucine, positively charged – lysine and arginine and also, hydrophobic amino acid tryptophan, that can bind to Zn^{2+} at the enzyme active site controlling the blood pressure (Marwa Yousr and Nazlin Howell, 2015).The ACE inhibitory peptide was found to be derived from tuna muscle having a novel amino acid sequence of Pro-Thr-His-Ile-Lys-Trp-Gly-Asp in which antepenultimate aromatic amino acid residue increases its binding capacity to ACE and hence, it further prevents its action with biological substrate.

3.4.2. Antimicrobial peptides

Antimicrobial peptides are the peptides which act directly to protect animals against a wide range of bacteria, fungi, viruses and protozoans i.e. has antimicrobial activity. They can further be distinguished on the basis of their amino acid composition and structure. There are different sources from which antimicrobial peptides have been isolated like dairy products, amphibian sources, marine fish sources and cereals. Out of these, marine fish sources have been found to have a high level of antimicrobial peptides which can be found to be used as food ingredients.

Comprehensive studies are done to demonstrate the antimicrobial activity of milk. Lactoferrin when hydrolyzed into lactoferricin in the gastrointestinal tract from milk act as an important precursor for the formation of many other bioactive peptides and itself shows potential antimicrobial activity. Lactoferrin in combination with milk Ig has synergistic action on micro-organisms. AMP's are isolated from casein, α- lactoglobulin and β-lactalbumin. It shows that the growth of *Listeria innocua, Micrococcus luteus, Salmonella enteritidisand E. coli* was inhibited by the isolated peptide (SSSEESII) from αs2–casein. IKHQGLPQE, a nanopeptide, withdrawn from casein hydrosylates was effective in decreasing the microbial load in infant formula (Kamali et al., 2017).

3.4.3. Antithrombotic

Antithrombotic means the prevention of formation or enlargement of blood clots in the body. Increased level of fibrinogen, hyperreactive platelets, irregular fibrinolysis lead to more chances of thrombotic activities. Antithrombotic drugs are given to increase fibrinolysis and reducing platelet aggregation, but drugs are always associated with their side effects. The mechanism involved in milk coagulation, in which there is interaction of k-casein with chymosin is remarkably identical to the mechanism involved in clotting of blood, characterized by the interaction of fibrinogen with thrombin. The k-casein fragment, casoplatelins, isolated from tryptic hydrolyzates, inhibits the activity of fibrinogen binding platelet, showing antithrombotic action. These peptides are released during gastrointestinal digestion and absorbed intact into the blood, which supports the concept that they exert an antithrombotic effect in vivo.

A study showed that bovine k-casein f106-f116, with amino acid sequence MAIPPKKNQDK, inhibited platelet aggregation and bind with the receptor site, further, preventing fibrinogen binding with blood platelets. The two more smaller tryptic peptides,

when isolated, (k-casein f106-f112 and f113-f116) did not exert much effect on platelet aggregation and were unable to inhibit fibrinogen binding. The behavior of k-casein f106-f116 is found to be identical to that of the C-terminal peptide of the human fibrinogen g-chain (Clare et al., 2000). The potential physiological effects of these antithrombotic peptides have not been yet established, but such peptides have been observed in the plasma of infants after breastfeeding or on ingestion of cow milk-based infant formula.

3.4.5. Osteoprotective

Osteoprotective literally means a bone protecting, i.e. the peptides which help in protection of bones in our body. To cure osteoporosis in our body, we need a higher intake of calcium in our diet which is bioavailable. It is found that casein and casein derived peptides have osteoprotective features, i.e. when compared to other proteins like whey protein, soy protein isolate, gluten, etc., casein peptides increased the intestinal calcium absorption in rats (17). Also, the study shows, colloidal calcium can easily form soluble complexes with casein phosphorylated peptides resulting in higher calcium retention. Studies on mini pigs when incorporated with casein peptides showed that changes in calcium absorption and metabolism could be examined under specific conditions only, i.e. when there is vitamin D or Ca deficiency, as compare to whey protein.

Whey components in the milk are found to increase the bone metabolism. These components are termed as milk basic proteins which are found to promote bone formation, bone mineral density, bone break force resistance, content of osteocalcin, but suppress bone resorption, pit formation and deoxypyridinoline levels in urine in animal and human model. Lactoferrin, on the other hand is found to be promoting factor for bones and anabolic factor in osteoporosis (17). Hence, both casein and whey proteins is effective to cure osteoporosis provided the diet of the person is adequate like Ca absorption was increased when diet was based on rice as staple food rather than whole grain.

3.4.6. Hypolipidemic and hypocholestrolenic

Any agent that reduces the level of lipids and lipoproteins in the blood is said to be hypolipedemic and they can be used in the prevention of accumulation of high levels of fats (cholesterol) in blood vessels. The studies have shown that high postprandial triglyceride levels in the blood can result in insulin resistance, atherosclerosis, obesity, etc. These postprandial triglyceride levels are reduced by decreased activity of the enzyme lipase and its

cofactor colipase which are required for the cleavage of dietary triglycerides and hence its absorption. Therefore, lipase inhibition results in preventing type 2 diabetes, accelerated weight loss and improving the metabolism. Method of hydrolysis of protein also determines whether the peptide will show antilipemic activity or not. It is reported that protease based hydrolysis result in increased lipid lowering activity while enzymatic hydrolysis lead to reduced lipid lowering activity of food derived proteins. Peptides isolated from soy protein, milk protein, buckwheat protein, egg white protein and fish protein show hypolipedemic and hypocholestrolenic activity.

A study conducted showed that prolamine (isolated from fish source), was able to reduce the triglyceride levels when incorporated in animals, but did not show any effect in human trial provided was given in varying amounts in liquid form which leads to the possibility that maybe, prolamine acts more effectively as lipase inhibitor when consumed in solid meals rather than in liquid form. Another study demonstrated effective lipase inhibitory activity by the peptides isolated from wheat flour, soybean cotyledon and defatted rice, when incorporated in rats. Also, rats supplemented with soy or fish protein hydrosylate had more high density lipoprotein to total cholesterol ratio than casein hydrosylates (17). The α subunit of soy 7S globulin was found to be responsible for a rise in low density lipoprotein degradation by effectively increasing the response in cultured hepatocytes for LDL receptor. CSP1, CSP2, CSP3 peptides isolated from cumin seeds repress cholesterol micelle formation and adhere to bile acids inhibiting the lipase activity results in hypocholestrolenic effect. Another study showed, like soy proteins, lupin proteins when hydrolyzed suppress the activity of HMG CoA reductase in HepG2 cells, accounting for the lowering of cholesterol in blood. There is a need to conduct more in vivo studies for commercial application of these peptides.

CHAPTER 4 RESEARCH GAP

Although, there are many sources from which bioactive peptides have been isolated, but present research is based on the novel plant/animal based source. Also, further studies are very limited on the functional properties of these peptides which will be fulfilled through this research. Incorporation of these peptides in different products can lead to development of variety of functional foods.

CHAPTER 5 OBJECTIVES

1. Optimization of process parameters for the extraction of protein.
2. Standardization of the process for the preparation of protein hydrolyzate
3. Purification, characterization and application of extracted peptides

Proximate analysis of the source :

Moisture content – AOAC 2000

Ash content - AOAC 2000

Protein content - Lowry method (Markwell et al., 1978)

Fat content - Ranganna 2016

Objective one: Optimization of process parameters for the extraction of protein.

- The production of bioactive peptides is to be carried out by enzymatic hydrolysis in which conditions to be optimized are enzyme to be used, temperature, time and pH for hydrolysis to occur.
- It is followed by centrifugation and any other membrane separation technique for the isolation of low molecular weight peptides.

Objective two: Standardization of the process for the preparation of protein hydrolyzate

- A particular time – temperature combination is standardized to isolate bioactive peptide from a desired source to prepare protein hydrozylate

Defatted source powder

⇩

Dispersed in base

⇩

Incubation

⇩

Centrifugation

⇩

Supernatant filtered with addition of acid

⇩

Centrifugation

⇩

Precipitates redispersed in base

⇩

Dried

Protein hydrosylate was checked for its protein content by lowry method.

- Enzymatic hydrolysis

Protein hydrosylate dispersed in acid

⇩

Addition of required enzymes

⇩

Standardizing pH and time – temperature

⇩

Incubation

⇩

Termination of enzyme reaction with addition of acid

⇩

Heating

⇩

Centrifugation

Objective three : Purification, characterization and application of extracted peptides

Purification is to be done by filtration followed by checking for its :

- Antioxidant assay - DPPH method, AOAC 2000
- Metal chelating activity – AOAC 2000

Also, amino acid profiling can be done with the use of HPLC and hence, can be incorporated into any food product to make it functional.

CHAPTER 7 EXPECTED RESEARCH OUTCOME

1. Isolation of peptide from novel plant source
2. The isolated bioactive peptides will posses functional properties.
3. Application of isolated bio-active peptide for new product development

CHAPTER 8 CONCLUSION

Bioactive peptides have a limited usage in the market or at the global level. Though, new technologies are emerging nowadays, to get the most active form of these peptides and incorporated to get the novel food product. The main focus needs to be given on bioavailabilty of these peptides after its intake. Further studies, in vivo and in vitro need to be performed to demonstrate its use on a social level for the prevention of various chronic diseases. Also, to increase the market of value- added products or functional foods globally, bioactive peptides act as a potential candidate.

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