ISOLATION AND CHARACTERIZATION OF BIOACTIVE PEPTIDES FROM CHIA SEEDS

M.Sc. PROJECT AND DISSERTATION - I

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DECLARATION

I hereby declare that the work presented in the dissertation- report entitled 'ISOLATION AND CHARACTERIZATION OF BIOACTIVE PEPTIDES FROM CHIA SEEDS' is my own and original. The work has been carried out by me at school of Agriculture, Lovely Professional University, Phagwara, Punjab, India; under the guidance of Er. Poorva Sharma, Assistant Professor (Food Technology) at school of Agriculture, Lovely Professional University, Phagwara, Punjab, India, for the award of the degree of Master of Science in food science and technology.

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CERTIFICATE

This is to certify that the thesis entitled **"ISOLATION AND CHARACTERIZATION OF BIOACTIVE PEPTIDES FROM CHIA SEEDS"** by **Roman Ahmadi** submitted to the Lovely Professional University, Punjab for the degree of Master of Science in food science and technology is a record of bona fide research work, carried out by them in School of Agriculture under my supervision. I believe that the thesis fulfils part of the requirements for the award of Master of Science in food science and technology. The results embodied in the thesis have not been submitted for the award of any other degree.

Date: 05/12/18

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1. INTRODUCTON

Bioactive Peptides (BP) have been defined as specific protein fragments that have a positive impact on body functions or conditions and may influence health (Kitts and Weiler, 2003). Currently, there are more than 1500 different BP have been reported in a database named 'Bio pep' (Singh *et al.*, 2014). BP are organic substances synthesized by amino acids and joined by covalent bonds also known as amide or peptide bonds, whereas proteins are polypeptides with a greater molecular weight (MW). BP and proteins play important roles in the metabolism of living organisms and, by extension, in human health (Korhonen and Pihlanto, 2006). They perform hormone or drug-like activities and can be categorized based on their mode of action as antimicrobial, antidiabetic, antithrombotic, antihypertensive, opioid, immunomodulatory, mineral binding and anti-oxidative respectively. The amino acid configuration (composition and sequence) determines the activity of the peptides once that they are released from the precursor protein where they are encrypted. Natural processes within the body are regulated by the interaction of specific amino acid sequences that form part of proteins. Proteins can be classified as endogenous if they are derived from amino acids synthesis within an organism, or exogenous if obtained through the diet or from an external origin to an organism, and they represent one of the primary components of the food. Proteins derived from plant and animal origins are potential sources of a wide range of BP coded in their structure (Shahidi, and Ying Zhong, 2008). Although the relationship between structure and functional properties is not yet well established, many BP share some similar structural features that include a peptide residue length ranging from 2–20 amino acids and the presence of hydrophobic amino acids in addition to proline, lysine or arginine groups.

Production of bioactive peptide by using lactic acid bacteria has been reported by Pessione. Lactic acid bacteria have been found to produce potential bioactive peptide with wide application in food and pharmaceutical industry.

1.1. Problem identified

With increasing consumer demands for less processed and more natural or functional foods, increasing efforts are focused towards the research on peptides and their use as food grade preservatives and functional food ingredients.

2. REVIEW OF LITERATURE

2.1. Bioactive peptide

Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). They usually range from two to twenty amino acids in length and have been derived from various plant and animal sources including milk, cheese, yoghurt, fish, soybean and kefir. Peptides derived from milk, in particular, have the greatest potential to be used commercially. Bioactive peptides derived from milk have been shown to have various properties including antimicrobial, antihypertensive, opioid, antioxidant, antithrombotic and mineral-binding.

2.1.1. History of Bioactive peptide

The first identified food derived bioactive peptides was discovered in 1950, when Mellander reported the casein phosphorylated peptides promoted vitamin D-independent bone calcification in rachitic infants. However, interest thing in this field has increased in the last two decades, in which most of the research rely on the identification of bioactive peptides from milk proteins. Bioactive peptides exist inactive form in the parent protein, it released in active after digestion of proteolytic enzymes (Korhonen *et al*., 2006). Methods employed in the proteolytic digestion of parent protein include: hydrolysis of by digestive enzymes, plant and bacterial proteases and following microbial fermentation (Rizzello *et al*., 2017).

2.1.2. Production and Processing of Food Protein-Derived Bioactive Peptides

From review of pertinent literature, the most common methods applied in the production bioactive peptides have been by enzyme hydrolysis of food proteins, fermentation or by chemical synthesis (which is mostly done for their purification and/or characterization). In few situations however, water extracts of mushrooms and some plant parts have proven to be direct sources of bioactive peptides.

Enzymatic activity: The most common way to produce bioactive peptide is enzymatic hydrolysis. Digestive enzymes and combination of different proteinases including acylase, chymotrypsin, pepsin and thermolysis as well as enzyme from bacterial and fungal source are used for the breakdown of protein.

Microbial fermentation: Several lactic acid bacteria (*Lactococcus lactis, Lactobacillus helveticus* have been reported to release bioactive peptide by the process of fermentation.

This method consists of a distinct intracellular peptidase includes endopeptidases, amino-peptidases, di-peptidases and tri-peptidases produced by the microorganisms during fermentation.

Comparatively microbial fermentation is the cheapest process instead of enzymatic hydrolysis for bioactive peptide production because microorganisms are a cheap source of proteases and recognized as safe. After the synthesis process, peptides are submitted to a separation procedure consisting of centrifugation and washing to remove residues of the reagents used, as well as products of side reactions. Subsequently, peptides are cleaved and subjected to filtration, as well as lyophilization. The most widely used methods used for the purification of peptides are reverse-phase high-performance liquid chromatography, ion-exchange chromatography, size exclusion chromatography, affinity chromatography, and capillary electrophoresis.

Methods of purification of peptides

Method	Principle	Uses
Reversed-phase chromatography	Based on hydrophobicity. Consisting of a stationary phase of lower polarity and a mobile phase of higher polarity	Enables rapid detection and purification of a peptide sequence from a mixture
Ion exchange chromatography	The distribution and surface charge of the peptide determines the interaction of charged groups with the surface of the stationary phase	Used for purification of peptides and proteins
Exclusion liquid chromatography	Based on separation process according to the size of the peptide relative to pore sizes in the stationary phase. Used primarily in the early stages of purification of the peptide, when performed in multiple steps	Used to separate low-molecular-weight impurities from a mixture of peptides. However, the separation of the peptide of interest with other closely related peptides is virtually impossible
Affinity chromatography	Based on the biological specificity of the peptide. Consists of a ligand (small specific biomolecule such as an antibody) that is immobilized in the column. The separation occurs because of highly specific biochemical interactions between the peptide and the ligand	Used when a high degree of specificity is required, for example, isolation of a target protein present in low concentration in a biological fluid or a cell extract

Capillary electrophoresis	Based on the migration of the peptide according to its charge in solution, depending on the application of an electric field. Complementary technique to reversed-phase chromatography	Used for peptides and proteins

2.1.3. Bioactive peptides and their roles in human health

Dietary proteins are traditionally known to provide a source of energy and the amino acids essential for growth and maintenance of various body functions. In addition, they contribute to the physicochemical and sensory properties of protein-rich foods. In recent years, food proteins have gained increasing value due to the rapidly expanding knowledge about physiologically active peptides. Chia seeds proteins provide a rich source of peptides which are latent until released and activated (Korhonen, Hannu, and Pihlanto, 2006). Functional of bioactive peptides are given in the following:

2.1.3.1. Antimicrobial activity

Antimicrobial peptides suppress the growth of microorganisms. Bioactive peptides having antimicrobial property possess potential applications in both food quality and safety and human health. In the last few years, considering the consumer demands for less processed and more natural or functional foods, increasing efforts are focused towards the research on peptides and their use as food grade preservatives and functional food ingredients (Brandelli *et al*., 2015).

Antimicrobial bioactive peptide derived from various source such as milk and egg have been reported to inhibit many Gram positive and Gram negative pathogenic strains such as *Escherichia coli*, *Aeromonas hydrophila, Salmonella typhii, Bacillus cereus, Salmonella typhimurium, Staphylococcus aureus* and many microbial infection (Mohanty *et al*., 2015). The mode of action of antimicrobial peptides has been extensively investigated and it has been observed that an amphiphilic, mostly alpha-helical formation, and an overall net positive charge is proposed to initiate the interaction with the bacterial surface and to enter the membrane (Floris *et al*., 2003). Cationic peptides are found to have an interaction with lipopolysaccharides present in cell wall of Gram negative bacteria resulting leakage of cellular material (Pritchard and Kailasapathy, 2011).

2.1.3.2. Anticancer activity

Cancer is a disease caused by uncontrolled division of abnormal cells in a part of body. Today cancer is the most predominant reason for the death of the person in all over the world. In 21th century due to the increased work load people not take care about their health which is the main reason of the generation of new kinds of diseases in which one of them is cancer which also associated with the consumption of wrong food. Traditionally to prevent chronic disease a person gone towards the expensive treatments like chemotherapy and radiations but now various alternatives are find out. Consumption of nutraceuticals and functional foods having ability to inhibit the chronic diseases. These foods contain some active components which are responsible for the curing of these diseases. Extraction of bioactive components from protein which derived from various plants and animal sources having potential to kill cancer cells which is a cost-effective method without any side effect. From all sources milk derived bioactive peptides (Proline and lysine) have much ability to fight against abnormal cells. Anticancer bioactive peptide display activities against cancer cells mainly by cytoplasmic membrane disruption through pore formation and apoptosis induction.

A cell selective peptide isolated from platensis hydrolysates showed strong inhibitory activity against cancer cell. peptides from tuna cooking juice have been shown to exhibit strong antiproliferative activity in breast cancer cell. In the same way, the peptide isolated from oyster hydrolysate showed anticancer activity against human colon carcinoma cell lines. Peptides from rapeseed protein fermentates have also been shown to inhibit the proliferation of human liver cancer, human breast cancer and human breast cancer cell lines.

2.1.3.3. Antidiabetic activity

Diabetes is a metabolic disease characterized by increased blood sugar level due to insufficiencies in insulin secretion, action, or both. The disease is classified into type I and type II. Type I diabetes (insulin dependent diabetes) is an autoimmune disease that causes the beta cells of the pancreas to secrete little or no insulin. In type 2 diabetes mellitus (T2DM), however, there is an imbalance in insulin secretion and blood sugar absorption. Current synthetic antidiabetic drugs may result in risks of hypoglycemia, weight gain, high background risk of pancreatitis and gastrointestinal side effects while some patients may not even tolerate them. For these reasons, the search for food derived anti-diabetic peptides is on the increase. Such alternatives may be safe as they are from food sources and have been consumed over the years without side effects. The peptides isolated from black bean protein hydrolysates effectively inhibited glucose transporter to reduce blood glucose levels.

2.1.3.4. Antihypertensive

Peptides Hypertension (high blood pressure) is characterized by a persistent systolic blood pressure (BP) value of ≥140 mmHg and a diastolic pressure of ≥90 mmHg (140/90). However, BP increases with age and hence only elderly people over 60 years with BPs above 150/90 mmHg may require treatment. Among the physiological mechanisms of hypertension, the renin-angiotensin system has attracted much scientific attention. Renin and angiotensin-converting enzyme (ACE) are the main enzymes involved in the renin-angiotensin system (RAS). Thus, the search for antihypertensive bioactive peptides from foods has increased. Food-derived antihypertensive peptides are known for their high tissue affinities and hence may be more slowly eliminated from tissues compared to synthetic drugs. To release antihypertensive peptides from whey, fermented whey from bovine milk with several Lactobacillus species which showed strong angiotensin converting enzyme inhibition. peptides from casein have been shown to significantly reduce high blood pressure in humans. Also, the peptides extracts from bamboo shoots has been shown to reduce high blood pressure.

2.1.3.5. Regulation of the gastrointestinal system

Food-derived proteins and peptides may play important functions in the intestinal tract before hydrolysis to amino acids and subsequent absorption. These include regulation of digestive enzymes and modulation of nutrient absorption in the intestinal tract. In the case of the latter functions, the role of bioactive peptides has been disputed for a long time.

2.1.3.6. Regulation of the nervous system

Peptides with opioid activity have been identified in various chia seeds proteins hydrolyzed by digestive enzymes. These opioid peptides are opioid receptor ligands with agonistic or antagonistic activities. Opioid receptors are located in the nervous, endocrine and immune systems as well as in the gastrointestinal tract of mammals and can interact with their endogenous ligands and with exogenous opioids and opioid antagonists. Thus, orally administered opioid peptides may modulate absorption processes in the gut and influence the gastrointestinal function in two ways: first, by affecting smooth muscles, which reduces the transit time, and second, by affecting the intestinal transport of electrolytes, which explains their anti-secretory properties.

2.1.3.7. Regulation of the immune system

Milk protein hydrolysates and peptides derived from caseins and major whey proteins can enhance immune cell functions, measured as lymphocyte proliferation, antibody synthesis and cytokine regulation. Of special interest are peptides released during milk fermentation with lactic acid bacteria, as these peptides have been found to modulate the proliferation of human lymphocytes, to down-regulate the production of certain cytokines and to stimulate the phagocytic activities of macrophages.

2.2. Functional foods

Increasing consumer awareness about the relationship between health and nutrition creates a supporting environment for the development of the functional food concept (Kechagia *et al*., 2013). Functional foods are defined as foods that improve the health of consumer or prevent the risk of diseases beyond its basic nutrition. They have also been referred as medicinal foods, nutritional foods, nutraceuticals, prescriptive foods, therapeutic foods, super-foods, designer foods and medicinal foods (Berner, O'Donnell, 1998). One way of creating a functional food is by inclusion of ingredients such as probiotics and prebiotics to levels that enable the consumer to derive optimal health benefits.

2.2.1. Probiotics

Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit on the host. Probiotics are commonly isolated from human and animal intestinal tracts. Dead bacteria and end product of bacterial growth also may transmit some health benefits but they are not considered as probiotics because they are not alive when administered.

Over the past two decades consumption of probiotic drink is increasing due to the awareness of people about the health benefits of these drinks (Ostlie *et al*., 2003). Probiotics can be consumed in any form but mainly they are marketed as capsules, powders, enriched yogurts, yogurt-like products and milks. Mainly used probiotics microorganisms are *Lactobacillus* spp. i.e. *L. acidophilus*, *L. casei*, *L. crispatus*, *L. gallinarum*, *L. gasseri*, *L. johnsonii*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus* and *Bifidobacterium* spp. i.e. *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. longum* (Holzapfel et al., 2001).

2.2.2. Desirable probiotic properties

In order to a potential probiotic strain following properties are expected:

- Acid and bile tolerance.
- Bile salt hydrolase activity.
- Antimicrobial activity against pathogenic microorganisms.
- Adhesion to mucosal and epithelial surfaces (Mercenier *et al*., 2008).
- Antimutagenic and anticarcinogenic properties.
- Antagonistic activity against pathogens such as *Helicobacter pylori*, *Salmonella* sp., *Listeria monocytogenes* and *Clostridium difficile* (Saarela *et al*., 2000).

2.2.3. Mechanisms of probiotic activity

Exact mechanism of their action is not yet known but they act by the following:

- Production of bacteriocin and short chain fatty acid.
- Lowering of gut pH.
- Nutrient competition to stimulation of mucosal barrier function and immunomodulation.
- Induce phagocytosis and IgA secretion.
- Modify T-cell response (Guarner and Malagelada, 2003, McNaught and MacFie, 2001, Isolauri *et al*., 2001)

2.2.4. Health benefits of probiotic bacteria

The microorganisms present in the colon act as a barrier to pathogenic microorganisms but these microorganisms diminish their integrity due to stress, illness, antibiotic treatment, changes in diet, or physiological alterations in the gut. Consumption of probiotics maintains the balance of microorganisms in the gut and prevents the risk of many diseases such as gastrointestinal disorders including gastrointestinal infections, inflammatory bowel diseases, and even cancer by reinforcing the body's natural defense mechanisms (Macfarlane and cummings, 1999, Saarela *et al*., 2000). Due to their health benefits probiotic bacteria have been increasingly included in yoghurts and fermented milks during the past two decades.

2.2.5. Probiotics as Diarrhea treatment

Rotavirus is the most common cause of severe diarrhea in the infants and young children. *Bifidobacterium* (which constitute the predominant intestinal flora of breastfed infants), as well as other lactic-acid-producing organisms such as *Streptococcus thermophilus* were found to have a protective effect against acute diarrhea disease (Saavedra *et al*., 1994). *S. thermophilus*, *L. bulgaricus*, *L. acidophilus,* and *B. bifidum* were also found to be effective against enterotoxigenic *E. coli* a causative agent to traveler diarrhea (Black *et al*., 1989). Probiotics microorganisms such as *L. rhamnosus, L. casei*, and the yeast *S. boulardii* was also found to reduced the risk of antibiotic associated diarrhea caused by *Clostridium difficile.*

2.2.6. Probiotics for lactose intolerance

Lactose intolerance is the inability to digest and absorb lactose (the sugar in milk) that results in gastrointestinal symptoms such as abdominal discomfort, flatulence and diarrhea when milk or food products containing milk are consumed. It is basically due to the deficiency of beta galactosidase enzyme which is required for the hydrolysis of lactose. Probiotic bacteria such as *S. thermophilus* and *L. delbrueckii* sp. *Bulgaricus* have high beta galactosidase activity and improve the metabolism of lactose in the lactose intolerant people (Kechagia *et al*., 2013).

2.2.7. Probiotics as allergy treatment

Probiotic bacteria also found to be effective against food allergen and atopic dermatitis. *B. lactis* and *L. rhamnosus* GG were found to be effective in decreasing the eczema severity in the infants. *L. rhamnosus* GG has been found successful in preventing the occurrence of atopic eczema in high risk infants, when supplied prenatally to selected mothers who had at least one first degree relative with atopic eczema, allergic rhinitis, or asthma.

2.2.8. Probiotic bacteria against cancer

Probiotic bacteria reduce the risk of cancer by decreasing the levels of carcinogenetic enzymes produced by colonic flora through normalization of intestinal permeability and microflora balance as well as production of antimutagenic organic acids and enhancement of the host's immune system (Hirayama and Rafter, 1999).

Other than these probiotic microorganisms also prevent the risk of coronary heart disease by reducing serum cholesterol level as well as blood pressure control (Sanders, 1999).

2.3. Antimicrobial activity of bioactive peptides

Antimicrobial peptides (AMPs), also called host defense peptides (HDPs) are part of the innate immune response found among all classes of life. Basic differences exist between prokaryotic and eukaryotic cells that may represent targets for antimicrobial peptides. The antimicrobial peptides (AMPs) are biologically active molecules produced by vast variety of organisms as an essential component of their innate immune response. The primary role of the AMPs is host defense by action cytotoxicity on the attacking pathogenic microorganisms, and they also serve as immune modulators in higher organisms. AMPs are considered as a promising and potential drug candidate for the future due to their wide range of activity, lesser toxicity, and decreased resistance development by the aim cells. The AMPs were found to exist in a broad range of secondary structures such as α-helices, β-strands with one or more disulphide bridges, loop and extended structures. The existences of such various structural forms of AMPs are very essential for their wide spectrum antimicrobial activity. Besides these properties, some crucial factors such as size, charge, hydrophobicity, amphipathic stereo geometry, and peptide self-association to the biological membrane also properties for their wide spectrum antimicrobial activity.The smaller size of AMPs facilitates the fast diffusion and secretion of peptide outside the cells, which is essential for extract immediate defence against action pathogenic microbes. The differences in the lipid composition between prokaryotic and eukaryotic cell membranes depute the aim for AMPs. The antimicrobial specificity of AMPs towards the aim cells was very dependent on the concessive interaction of peptides with the microbial cells, which made powerful them to kill specific aim cells without affecting the host cells. Also, pure charge and hydrophobicity of AMPs play a vital role in cellular community of these peptides to selective target cellular membranes in action antimicrobial activity (Meyer and Nes, 1997). AMPs have been obtained from different sources such as plants, mammals, animals, microorganism, maritime invertebrates, and environmental libraries. Currently, more than 2,000 AMPs have been stated in antimicrobial peptide database. Most of them are cationic peptides, and only a few of them are anionic, which shared the ability to fold into amphipathic combination upon interacting with the membranes. Antimicrobial function, AMPs also serve as drug transfer

vectors, antitumor agents, mitogenic agents, contraceptive agents, and signalling molecules in emblem transmission paths. This review enable realization into antimicrobials as well as multifunctional properties of AMPs that provides better understanding of multipurpose biological properties of AMPs for prophylactic and remedial usage. Over the past 40 years, a number of extra antimicrobial substances produced by epithelia and phagocytes have been specified, arrange in size from small inorganic molecules such as hydrogen peroxide to large protein complexes such as those generated by the activation of the complement cascade. Antimicrobial peptides are formally defined as polypeptide antimicrobial substances, encrypted by genes and synthesized by ribosomes, with fewer than 100 amino acid remains. This definition recognized them from most (but not all) peptide antibiotics of bacteria and fungi, which are combined by specialized metabolic paths and mostly synthesize exotic amino acids (Brogden, 2005).

2.3.1. Structure of AMPs

Antimicrobial peptides are exclusive and various group of molecules, which are separate into subgroups on the base of their amino acid composition and structure. Antimicrobial peptides are generally between 12 and 50 amino acids. These peptides include two or more positively charged remainder prepared by arginine, lysine or, in acidic surroundings, histidine, and a large proportion (generally >50%) of hydrophobic remainder. The secondary structures of these molecules follow 4 themes, including

1. α-helices
2. β-strands due to the presence of 2 or more disulphide bonds
3. β- hairpin or loop due to the presence of a single disulfide bond and/or cyclization of the peptide chain
4. Extended

Many of these peptides are unstructured in free solution, and disarrange into their final configuration upon partitioning into biological membranes. It contains hydrophilic amino acid remains equal along one side and hydrophobic amino acid remains equal along the opposite side of a helical molecule. This amphipathic of the antimicrobial peptides lets them to partition into the membrane lipid two layer. The capability to relation with membranes is a definitive feature of antimicrobial peptides however membrane permeability is not necessary. These peptides have a diverse of antimicrobial activities ranging from membrane permeability to action on a range of cytoplasmic targets (Meyer and Nes, 1997).

2.4. chia seeds

Chia (Salvia hispanica L.) is an annual herbaceous plant that belongs to the Lamiaceae family. This plant is native from southern Mexico and northern Guatemala and has recently been marketed as a crop in South America. The use of chia may be in the form of whole seeds, flour, mucilage and oil seed. The chia seed has been described as a good source of oil, protein, dietary fiber, minerals and polyphenolic compounds (Ayerza, 1995). In addition, chia seed and oil contain a rich pool of natural antioxidants such as tocopherols, phytosterols, carotenoids and phenolic compounds, including chlorogenic acid, caffeic acid, myricetin, quercetin and kaempfero which protects consumers against many diseases and also promotes beneficial effects on human health. Chia seed and oil are important raw materials to functional foods due to its bioactive components (da Silva Marineli, Rafaela, et al, 2014). Chia grain has several features which make it very competitive with the other grain sources. The chia seed has more than one and one half to twice the protein concentration of other grains and approximately three to ten times the oil concentration of most grains. Its high protein and oil concentration makes it a very attractive grain source for developing countries which have a shortage of protein and energy. However, its high fiber concentration would limit its usage to adult diets (Weber, Charles, et al, 1991). The benefits of chia seed has recently been described for human health and nutrition because their bioactive components were found to promote health benefits , improving biological markers related to dyslipidemia, inflammation, cardiovascular disease, glucose homeostasis, and insulin resistance, without promote adverse effects. Geographical origin is known to have significant impacts on seed composition and concentration of bioactive compounds (Ayerza & Coates, 2011). Although plants are little frost-tolerant, they can be grown in green houses in some parts of Europe. Today, chia is grown commercially in Mexico, Bolivia, Argentina, Ecuador, and Guatemala. In Argentina, it is a summer-autumn crop that could be grown economically instead of non-profitable traditional crops in the north-western region. Chia seeds have long been used by the Aztec tribes. They are important not only as food, but also for medicines and paints. It is traditionally consumed in Mexico and the southwestern United States, in a minor extent in South America, though is not widely known in Europe. They have been investigated and recommended due to their content of oil, protein, antioxidant and dietary fiber content. The plant is day light sensitive and produces small white and dark seeds. Their shape is oval, measuring 2.0mm× 1.5mm. Most of chia populations grown today contain a low percentage of white seeds. This type of seeds come from plants that produce only white seeds, which are

encoded by a single recessive gene. In general, there is a little size difference between these seeds, white seeds are larger than the black ones. Also, there are some differences in protein content and fatty acid composition between dark and white chia seeds extraction. Thus, the knowledge on the morphology and size distribution of chia seeds is essential for an adequate design of the equipment for cleaning, grading and separation, moisture content for the development of the drying process, gravimetric properties for the design of equipment related to aeration, drying, storage and transport. Bulk density determines the capacity of storage and transport systems while true density is useful for separation equipment, porosity of the mass of seeds determines the resistance to airflow during aeration and drying. Frictional properties, such as the angle of repose and the coefficient of external friction, are important for conveying systems, the design of grain bins and other storage structures whose operation is influenced by the compressibility and flow behaviour of materials (Ixtaina, Vanesa, Susana, Nolasco, and Mabel 2008). These plants are low water users and well adapted to arid climates. The chia species grows as either summer or winter annuals. The summer annuals are Hyptis suaveolens and Salvia hispanica while the winter annuals are Salvia columbariae and Salvia carduacea. The two groups of chias are adapted to two distinct environments of the summer annual rain climate or to the winter annual Mediterranean type of climate.

Different types of chia seeds with different protein, oil and ash content.

Source	Protein %	Oil %	Ash %
Hyptis suaveolens	18.7 - 22.3	13.5 - 33.6	4.0 - 8.1
Salvia columbariae	19.3 - 24.0	31.6 - 34.4	5.3
Salvia carduace	26.0 - 26.5	30.0 - 32.1	5.9
Salvia hispanica	18.0 - 23.6	24.3 - 34.1	4.6 - 5.1

According to that table chia had a range of protein from 18.0 to 26.5%, oil from 13.5 to 34.4%, and ash values from 4.0 to 8.1%. The ash content of chia was fairly constant. However, the protein and oil content had wide variations.

3. OBJEVCTIVES

- Isolation of bioactive peptides.
- Antimicrobial activity of bioactive peptides.
- To optimize the extraction of gum from chia seeds.
- To analyze the Physico-chemical properties of chia seeds.

4. MATERIALS AND METHODS

4.1. Chemical analysis

Moisture, fat, protein, were determined. Protein was determined by the Kjeldahl method and Total lipids were determined by the method of Gerber and moisture in an oven at 100 - 105 °C.

4.2. Plastic Ware and Glass-wares

Storage bottles, micro-centrifuge tubes etc. were purchased from Tarsons. Petri dishes were procured from oxygen. Measuring cylinders, beakers, conical flasks, glass bottles, test tubes etc. were procured from M/s. Borosil Prior to use, all the glass wares were cleaned by washing with a mild detergent followed by rinsing with tap water and finally with distilled water and autoclaved at 121 ºC, 15 psi for 15 minutes. Micropipettes (Eppendorf), syringes (Dispo Van), syringe filters (Millex GV), vials, glass pipettes, aluminum foil, and parafilm, etc. were also used.

4.3. Growth medium, microorganisms and culture conditions

MRS-Cysteine medium (M369) was used as growth medium for probiotic strains. Growth medium was prepared as per manufacturer instructions followed by sterilization by autoclaving at 15 psi for 15 min prior to use. Lyophilized culture of probiotic strain used in this work was obtained from Imtech Chandigarh. Chia seeds were obtained from local market of Jalandhar. All microbial samples and sterile media were carefully handled using standard aseptic laboratory techniques under bio-safety cabinet to maintain sterility.

4.4. Equipment

The equipment used during the present study includes Electronic balance (CONTECH), pH meter (LABTRONICS), Autoclave (NSW), Incubator shaker (NSW), Centrifuge (REMI), Microwave (LG), Vortex (Tanco), UV/Visible Spectrophotometer (Agilent), Magnetic stirrer (Remi), Mixer grinder (USHA), Water bath (Tanco) and Refrigerator (LG) etc.

4.5. Preparation of MRS-Cysteine medium

MRS broth was prepared as per manufacturer's instructions and autoclaved at 121°C, 15 psi for 15 min. After autoclaving, medium was cooled down to the room temperature and then 1 % (v/v) of filter sterilized (0.22 µm) Cysteine HCL was aseptically added to the prepared MRS medium and mixed carefully to avoid any gas bubble formation.

4.6. Optimization of water for chia seeds gum extraction

Seeds were soaked in distilled water in a 1:20 (g:mL) ratio for 2 h to allow gum production, then filtered through double layer cheese muslin cloth to separate gum from seeds and Covered beaker of gum with aluminium foil and kept in the water bath at 85 °C for 30 min gel is formed. Than removed from water bath and kept in the other beaker which filled with cold water (2 – 4 °C) for 12 hr and stored at refrigeration condition prior to use.

4.6.1. Physico-chemical analysis of chia gum

Determination of Moisture Content

Chia gum (2ml) each was dried in a clean, dry and pre-weighed petri dish and kept in with lid open at 100-105°C for 1-3 hours in hot air oven till constant weight. After cooling in desiccator, loss in weight was calculated as moisture of sample and expressed as per cent moisture.

$$\text{Moisture \%} = \frac{\text{Weight of fresh sample(g)} - \text{Weight of dried sample(g)}}{\text{Weight of fresh sample(g)}} \text{x} 100$$

Titratable Acidity

The TA was determined by titrating 2ml of chia gum with 0.1 N NaOH using phenolphthalein as an indicator and the results were expressed as the percent of lactic acid by using the following formula:

$$\text{TA\%} = \frac{\text{Titre value} \times \text{N of NaOH} \times 0.064 \times 100}{\text{Volume of sample}}$$

Where *TA*= titratable acidity

N= normality of NaOH

0.064= acid equivalent (Ranggana, 1977).

PH

PH of the sample was checked with calibrated digital pH meter (Orion).

Fat test

Fat was estimated by using Gerber method by using Butyrometer.

TSS (total soluble solid)

Total solids were determined by subtracting the moisture %.

Protein Test

Protein content of the sample was determined according to the principle of the Kjeldahl method. The sample (1 g) was digested with 15 mL of concentrated sulphuric acid, using an electrically heated aluminum block digester. The resulting digest was diluted and then made alkaline with 50 mL 40% sodium hydroxide. This was followed by rapid steam distillation of ammonia from the diluted digest into 25 mL of 4% boric acid for manual titration with 0.2 N hydrochloric acid.

$$\text{Amount of nitrogen present in the sample} = \frac{(y - v) \times 0.00014 \times 50}{\text{weight of sample}} \times 100$$

$$\%\ \text{protein content in the sample} = \frac{6.25 \times (y - v) \times 0.00014 \times 50}{\text{weight of sample}} \times 100$$

Titre volume of sample = (y – v)

Total volume made from digested sample = 50 ml

4.7. Proteolytic activity assay

Qualitative assay

Proteolytic activity of probiotic strains was examined by the method given by Beganovic *et al*., 2013. Agar well diffusion method by using skim milk agar was used for the assay. Actively grown culture of probiotic strains were placed in the centre of skim milk agar plate

and incubated at 37°C for 24h. After 24h incubation absence or presence and diameter of zone of inhibition around the well was checked.

4.7.1. Growth of probiotic strains in MRS-Cysteine medium

MRS-Cysteine medium was used for the revival of lyophilized probiotic cultures. In order to understand the growth pattern of probiotic bacteria in MRS-Cysteine medium, static fermentation was carried out for 24 h at 37 °C.

Procedure

Seed culture was prepared by inoculating lyophilized culture (1%) of probiotics in 70 ml MRS-Cysteine broth in 100 ml schott bottle and then incubated statically at 37°C for 24 h.

1. 10% (v/v) of primary seed culture was then transferred into 70 ml secondary seed culture (MRS-Cysteine broth) and cultivated for 24 h at 37°C in static condition.
2. Secondary seed culture (10%, v/v) was again transferred to 70 ml MRS-Cysteine broth and incubated at 37°C for 24 h and designated as tertiary seed culture. Optical density was measured at 600 nm.

4.8. Preparation of chia seeds fermentation medium

Chia gum was sterilized by pasteurizing at 80ºC for 30 min in water bath. After pasteurization, sterility of fermentation medium was then checked by spreading small volume on nutrient agar (NA) to check the presence of bacteria.

4.9. Seed culture preparation for chia seeds fermentation medium

Probiotic bacterial inoculum was prepared from the static fermentation of MRS-Cysteine broth till tertiary seed culture for 14 h as described above. After that, whole cell culture fluid was centrifuged at 10,000 rpm for 10 min. Cell pellet was washed twice with sterile saline solution (0.85% sodium chloride) and finally dissolved in equal volume of chia gum and incubated at 37°C for fermentation. During fermentation gum was analyzed for change in pH, TSS, antioxidant and proteolytic activity.

4.10. Proteolysis assessment of fermented chia gum medium

Proteolytic activity of probiotic bacteria in the fermented sample was determined by using the *o*-phthalaldehyde (OPA) test. The increase in optical density at 340 nm relative to the control was determined by using the spectrophotometer (Pescuma *et al.*, 2010).

4.10.1. Principle

α – Amino group released by hydrolysis of protein react with *o*-phthalaldehyde and 2-mercaptoethanol to form an adduct that absorbs strongly at 340 nm. The absorptivity is similar for all α – amino groups.

4.10.2. Preparation of OPA solution

For the preparation of 50 ml OPA solution 2.5 ml of 20% (w/v) SDS (Sodium Dodecyl Sulphate), 25 ml of 100 millimolar/l sodium tetraborate, 40 mg of OPA dissolved in 1 ml of methanol, 100µl of 2-mercaptoethanol was mixed properly and final volume was made up with distilled water.

4.10.3. Standard curve of L-leucine

For the preparation of standard curve of L-leucine, a stock solution of leucine containing 0.02 g leucine in 10 ml was prepared. Different concentrations of L-leucine covering the range of 2 - 10µl of stock were prepared in addition to a blank that did not contain L-leucine. Each sample volume was made up to 50µl by using distilled water and mixed with 1.0 mL of OPA solution. The samples were incubated at room temperature for 5 minutes. The absorbance was determined at 340 nm using spectrophotometer.

4.10.4. Preparation of sample for proteolysis assessment

For the preparation of sample for proteolysis assessment, fermented chia gum was incubated with 0.75mol/l trichloroacetic acid (1:3) at 4ºC for 30 minutes and centrifuged (5000 rpm, 10 min). Supernatant was used to check the proteolytic activity.

4.10.5. Determination of proteolytic activity

50µl of obtained supernatant of sample remove was mixed with 1 ml of OPA solution and incubated at room temperature for 5 min. Optical density was taken at 340 nm. Proteolytic activity was expressed as micro gram leucine released per ml by using the standard curve of L-leucine.

4.11. Hydrolytic assessment of fermented chia seeds medium

Proteolysis of fermented sample was determined by SDS-PAGE electrophoresis. Fermented and non-fermented chia gum was first treated with SDS (10%) for 10 min at 90ºC temperature, centrifuged at 10.000 rpm for 10 min and then supernatant was collected and 2 µl of each sample was prepared separately in denaturing 4X buffer and heated at 100 °C for 5min before electrophoresis. The gels were run in a Tris-glycine buffer and the electrophoresis was carried out at a constant current of 50 mA. After electrophoresis, proteins in gels were stained with Coomassie Brilliant Blue R-250.

4.11.1 Principle

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively. It is particularly useful for monitoring protein purification, and, because the method is based on the separation of proteins according to size, the method can also be used to determine the relative molecular weight of proteins. SDS is an anionic detergent. Samples to be run on SDS-PAGE are firstly boiled for 5 minutes in sample buffer containing 2-mercaptoethanol and SDS. The 2-mercaptoethanol reduces any disulfide bridges present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the SDS molecules. The sample buffer also contains an ionizable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well. Once the samples are loaded a current is passed through the gel. The samples to be run are not in fact loaded directly into the main separating gel. When the main separating gel has been poured, a shorter stacking gel is poured on top of the separating gel. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. Typically, the separating gel used is a 15% polyacrylamide gel.

The relative molecular weight of a protein can be determined by comparing its mobility with those of a number of standard proteins of known molecular weight that are run on the same gel.

4.11.2. Materials

- Gel Apparatus
- Gel Assembly (Glass plates, gasket, spacer, holder etc.)
- MilliQ water
-

4.11.3. Reagents

- 29% Acrylamide/1.0% Bis-acrylamide
- 1.5M Tris base (pH 8.8)
- 0.5M Tris base (pH 6.8)
- 10% SDS
- 10% APS
- TEMED
- 4X Sample buffer
- 5X running buffer
- Coomassie Blue R-250
- Methanol
- Acetic acid

4.11.4. Reagents preparation for SDS PAGE

1. **30% Acrylamide/0.8% Bis-acrylamide**
    - 29.0 gm Acrylamide

- 1.0 gm Bis-acrylamide
- 100ml MilliQ

2. Tris-HCl, pH 8.8

- 22.72gm Tris base
- Dissolved in 60ml MilliQ
- Adjust pH to 8.8 with 5NHCl
- Dilute to 100ml

3. Tris-HCl, pH 6.8

- 15.12gm Tris base
- Dissolved in 60ml MilliQ
- Adjust pH to 6.8 with 5N HCl
- Dilute to 100ml

4. 10% SDS

- 10gm SDS
- 100ml MiiliQ

5. 10% APS (Ammonium per sulfate)

- 0.5gm APS
- 5ml MilliQ

(Prepared fresh after 2 days)

6. Running/Electrophoresis Buffer (5X), pH 8.3

- 15.1gm Tris-HCl

- 72gm glycine
- 5gm SDS
- 1000ml MilliQ

(Dilute to 1X before use)

7. Sample Buffer (4X)

- 2.5ml Tris-HCl, pH 6.8.
- 4 ml glycerol.
- 0.8ml 10%SDS.
- 4 ml 2-mercaptoethanol.
- 2 mg Bromophenol blue.

(Composition for 10 ml)

8. Staining solution

- 0.25gm Coomassie brilliant blue R-250
- 125ml Methanol
- 25ml Acetic acid
- 100ml MilliQ

(Composition for 250 ml)

9. Destaining solution

- 45ml Methanol
- 10ml Acetic acid
- 45ml MilliQ

4.11.5. Procedure

- Assemble the glass plates, sandwich of the electrophoresis apparatus using two clean glass plates and two 0.75mm spacers. Lock the sandwich to the casting frame.
- Prepare the Separating Gel (15%)- for 10 ml
    - ✓ Acrylamide/Bisacrylamide = 5.0ml
    - ✓ Tris HCl pH 8.8 = 2.5ml
    - ✓ 10% SDS = 0.1ml
    - ✓ MilliQ = 2.3ml
    - ✓ APS = 0.1ml
    - ✓ TEMED = 10µl

  Pour the separating gel to the sandwich along an edge of one of the spacers.

  Slowly cover the top of the gel with around 1cm of water saturated butanol. Allow the gel to polymerize for 15 minutes.

- Pour off butanol and rinse completely with MilliQ.
- Prepare the Stacking Gel (4%)- for 5 ml
    - ✓ Acrylamide/Bisacrylamide = 0.85ml
    - ✓ Tris HCl pH 6.8 = 0.626ml
    - ✓ 10% SDS = 50µl
    - ✓ MilliQ = 3.422ml
    - ✓ APS = 50µl
    - ✓ TEMED = 5µl

  Stacking gel solution was poured over separating gel. Insert a 0.75mm Teflon comb. Allow the gel to polymerize for 15minutes.

- Dilute an aliquot of protein sample with 4X sample buffer and incubate for 5 minutes at 100°C.
- After removing the Teflon comb the wells were filled with 1X running buffer. Using a 25 or 100µl pipette with a flat tipped needle, samples were applied in the wells. Control wells were loaded with markers.
- Whole chamber was then filled with 1X running buffer.

Power supply was connected and run at 50 mA constant current for 180 minutes, then the power supply was switched off and the gel was stained in staining solution for 2 hours on shaker and then destained overnight on shaker.

4.12. Antimicrobial activity:

Agar well diffusion method was used to evaluate the antibacterial activity of fermented milk against *Bacillus subtilis, Staphylococcus aureus* and *E. coli*. After 48h of fermentation, fermented milk was centrifuged at 10,000 rpm for 15 min and filter sterilized by using syringe filter of 0.22µm. Nutrient agar plates were seeded with vegetative cells of test bacteria (50 µl) and then 8.0 mm well was punched in the center of plate by using a sterile cork borer. 100 µl of fermented sample was then added to the wells separately along with non-fermented sample as control. All the agar plates were incubated at 37°C for 24 h and zone of inhibition was observed.

5. CONCLUSION

Bioactive peptides possess potential applications in both food quality and safety, and human health. Considering the consumer demand for less processed and more natural or functional foods, increasing efforts was focused towards the research on peptides and their use as food grade preservatives and functional food ingredients.

Therefore, as per study we can conclude that lactic acid bacteria are able to grow on flaxseed milk and release bioactive peptides during fermentation. These peptides have several bio functional activities including antimicrobial, antioxidant, antihypertensive etc. Antimicrobial activity of bioactive peptide mainly depends on their amino acid chain. Therefore, there is need to investigate sequence of amino acid those act as pathogens. There is also need to study exact mechanism of action of these peptides.

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