‘ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPONENT FROM SOYBEANS''

Dissertation I

Submitted by

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CERTIFICATE

This is to certify that Ahmed Mohamed Dahir **(Registration NO. 11700473)** has personally completed M.Sc. dissertation I entitled **'isolation and characterization of bioactive component from soybeans''**", under my guidance and supervision. To the best of my knowledge, the
present work is the result of hid original investigation and study. No part of dissertation-1 has ever been submitted for any other purpose at the university. The project report is appropriate for the submission and the partial fulfillment of the conditions for evaluation leading to the award of Master of Nutrition and Dietetics

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DECLARATION

I hereby declare that the work presented in the dissertation entitled **'isolation and characterization of bioactive component from soybeans'** is my own and original. The workhas been carried out by me at school of Agriculture, Lovely Professional University, Phagwara,Punjab, India; under the guidance of **Er.Poorva**, Assistant Professor (Food Technology) at school of Agriculture, Lovely Professional University, Phagwara, Punjab, India, for the award ofthe degree of Master of Science in Nutrition and Dietetics.

Date: May, 2018 Ahmed Mohamed Dahir (11700473)

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

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Introduction

Bioactive peptides have determined protein fragments that has a positive effect on the body functions and can ultimately be beneficial to health (kitts, D.D et al.2003).

According to Haque et al. (2009), bioactive peptides derived from milk play an important role in human's health and nutrition Many researchers are interested in solving the question of meaning of bioactive foods are made up of food or drugs and careful consideration is required.

Once bioactive peptides are released, they may act as regulatory compounds with hormone like activity. This aspect has been studied since 1979 and numerous peptides, which exhibit various activities such as opiate, antithrombotic or anti-hypertension activity, immunomodulation or mineral utilization properties, has been found. Milk proteins are the most important source of bioactive peptides, though other animal as well as plant proteins also contain potential bioactive sequences.

The first biologically active peptide discovered in milk was opioid peptides followed by immunomodulatory peptides.

In according to the fitzGerld and Murry, Bioactive peptides have been described as peptides which act same as hormone or drug like activity that ultimately changes the physiological functions ofthe body according to their activity (FitzGerld R.et al.2006).

Bioactive peptides are existing in a number of processed and fermented foods, their true physiological functions in humans are unknown. In healthy individual, eating a varied diet,the presence of bioactive peptides may help keep the nervous, immune and digestive systems in a well-maintained state.

The future potential value of bioactive peptides in the diet may be their ability to affect certain pathological conditions, although this has yet to be proven.

Casein derived peptides have already found interesting applications as dietary supplements (phosphopeptides) and as pharmaceutical preparations (phosphopeptides, β-casomorphins).

The efficacy and safe conditions of use of these peptides in animals and in humans remain to be proven. At present, ACE-inhibitory peptides and phospho-peptides are an important area in which bioactive peptides may be found to be useful ingredients for dietary applications. (Brulé et al. 1982).

History of bioactive peptides

The first identified food derived bioactive peptides was discovered in 1950, when Mellender reported the casein phosphorylated peptides promoted vitamin D-independent bone calcification in rechetic infants.(Mellander et al.1950).

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 (Rutherfurd-Markwick et al.2005).

Bioactive peptides exist inactive form in the parent protein, it released in active after digestion of Proteolytic enzymes (Korhonen et al.2009).

Methods employed in the proteolytic digestion of parent protein include: hydrolysis of by digestive enzymes (Hernandez-Ledesma et al.2007), plant and bacterial proteases (Zhu et al.2006).and following microbial fermentation (Rizzello et al.2005).

The most widely reported bioactive peptides from milk and other food sources display antihypertensive activity, particularly those peptides which inhibit the action of angiotensin-1-converting enzyme (Li, G.H. et al. 2004). Indeed, currently a number of commercial products which contain such peptides have been released onto the market.

Perhapsthe two most widely known products available are both fermented milk-based products, example Calpis and Evolus.

Calpis is a sour milk product from Japan which contains the antihypertensive peptides VPP and IPP both derived from milk caseins.

Evolus is a Finnish product that claims to reduce blood pressure.

Evolus also contains the peptides VPP and IPP which correspond to the fragments of β-casein f (Katayama et al.2003), and κ-casein f. Various milk protein hydrolysates which claim to contain bioactive peptides are also available as food ingredients. For example C12®, a casein derived peptidesupplied by DMV International, claims to reduce blood pressure. (Hayes et al.2007).

Sources of Bioactive peptides

Among the macronutrients present in foods, peptides and proteins are of paramount importance, because they supply the required raw materials for protein biosynthesis and represent a source of energy (Dziuba, 2014k et). Also, they are part of an intricate series of organic transformations that occur during the processing and storage of foods that ultimately contribute to their sensory characteristics. In addition to their nutritional value, food proteins, and peptides exhibit distinct biological activities (Hartmann and Meisel, 2007; Moller et al., 2008).

BP are predominantly encrypted inside bioactive proteins(Meisel and Bockelmann, 1999). By far, bovine milk (Torres-Llanezet al., 2005; Korhonen, 2009; Léonil, 2014; Mohanty et al., 2015;Mohanty et al., 2016), cheese (Pritchard et al., 2010), and dairy products(Choi et al., 2012) are the greatest sources of bioactive proteins and peptides derived from foods.

However, they can also be obtained from other animal sources such as bovine blood (Przybylski et al.,2016), gelatin (Lassoued et al., 2015), meat, eggs, various fish species such as tuna, sardine, herring and salmon. Some vegetal sources of BP and proteins are wheat (Kumagai, 2010), maize, soy (Singhet al., 2014), rice (Selamassakul et al., 2016), mushrooms, pumpkin,sorghum (Moller et al., 2008), and amaranth (Silva-Sanchez et al.,2008). In vivo, encrypted peptides can be liberated during gastrointestinal(GI) digestion by enzymes such as trypsin or by microbialenzymes. In vitro, BP can also be released during food processing or ripening by microbial enzymes (e.g. Lactobacillus helveticus) (Gobbetti et al., 2002; Meisel, 2005; Korhonen and Pihlanto, 2006;Korhonen, 2007; Dziuba and Dziuba, 2014). BP have been identified and isolated from animal and vegetal sources and are abundantly present in protein hydrolysates and fermented dairy products. Currently, BP and nutraceutical proteins are being developed to improve human health by preventing or alleviating medical conditions such as coronary heart disease, stroke, hypertension, cancer,obesity, diabetes, and osteoporosis (Gilani et al., 2008; Boelsma andKloek, 2009).

Animal sources

Peptides derived from animal proteins have been attributed to different health effects (Bhat et al. 2015b). Blood is a valuable source of proteins (circa 20%) and represents a promising source of BP.Although blood disposal is a severe problem for meat processors,serum albumin, the main blood protein has received little attention.

In a recent study, serum albumin was hydrolyzed using different concentrations of trypsin and the peptide sequences in the hydrolysates presented the following activities: angiotensin-converting enzyme(ACE) inhibition (antihypertensive activity), DPP-IV inhibition (glucose regulation), and antioxidation (Arrutia et al., 2016a). Blood obtained from the slaughterhouse is part of the meat production food chain that has not been fully exploited. In a recent study, bovine haemoglobin from the slaughterhouse, blood was subjected to in vitro GI digestions, 75 unique peptides were unambiguously identified using low-resolution (LR) liquid chromatography(LC)-MS/MS analysis.

The use of high-resolution (HR) liquid chromatography (LC)-MS/MS allowed identifying more than 950 unique peptides (Caron et al., 2016). Haemoglobin fragments can have a profound physiological function. The α- and β-globin chains of haemoglobin provide relatively long peptides containing ca. 30 amino acid residues upon proteolysis.

Degradation step coupled with excretion afforded shorter peptides from red blood cells. Both the primary and the secondary proteolysis products were subjected to further stepwise C- and N-terminal chain shortening, giving rise families of closely related peptides that are found in animal tissue extracts (Ivanov et al., 1997).

Milk

Bovine milk, cheese, and dairy products are the best sources of bioactive proteins and peptides derived from food (Korhonen, 2009; El-Salam and El-Shibiny, 2013; Lemes et al., 2016; Mohanty et al., 2016).

Presumably, this may be one of the primary reasons why milk is required beyond nutrition in the first months of life (Moller et al., 2008).

Milk proteins have a range of biological activities. For instance,immunoglobulins have an immunoprotective effect and lactoferrin (Lf) displays antibacterial activity. Low concentrations of growth factors and hormones, mainly present in colostrum, appear to play a significant role in post-natal development (Park and Nam, 2015).

The major role of milk proteins is to supply amino acids and nitrogen to the young mammals and constitute an important part of dietary proteins for the adult (Sharma et al., 2011). Milk proteins are a rich source of biologically active peptides that are released during GI digestion or food processing (Fitzgerald and Meisel, 2003).

As an example, opioid peptides that exist in dairy products have pharmacological properties similar to morphine and play an active role in the central nervous system (CNS) (Haque et al., 2008).

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Using liquid chromatography-mass spectrometry (HPLC-MS) and tandem mass spectrometry (MS), a large number of medium and low-MW BP (opioid, phosphopeptides) were identified in human milk from mothers of pre- and full-term infants. The formation of many peptides confirms the greater susceptibility of human milk to casein proteolysis compared to bovine milk. Characterization of the peptide sequence, allowed to establish the pathway of casein hydrolysis which leads to the formation of small peptides. It was found that the action of a plasmin-like enzyme acting on specific lysine residues is the primary step in casein degradation. This step is followed by endopeptidases and/or exopeptidases mediated cleavage of the oligopeptides to produce a multiplicity of short peptides differing by one or more amino acid residues. This work reinforces the importance of maternal milk and demonstrates the difficulty to reproduce it artificially. As a consequence of the dynamic nature of maternal milk, a succession of potentially BP is produced in the intestine, which is hard to reproduce in artificial products. Further studies are therefore required to ascertain the role of peptides derived from casein as nutritional and pharmacological factors (Ferranti et al., 2004).

Egg

Eggs are known as a source of valuable proteins in human nutrition and have been considered an important source of many BP (Wu et al.,2010; Zambrowicz et al., 2011; Bhat et al., 2015a) which may find applications in medicine and food industry (Sun et al., 2016).

The identification and characterization of biologically active peptides released in vitro or in vivo from egg proteins have been achieved, and the results have contributed to change the image of the egg as a new source of biologically active ingredients for the development of functional foods with specific benefits for human health and the treatment and prevention of diseases (Bhat et al., 2015a).

It is now well established that eggs contain numerous substances with potential and demonstrated therapeutic effects, beyond supplying basic nutritional requirements (Zambrowicz et al., 2011). The BP Arg-Val-Pro-Ser-Leu obtained from egg white protein was chemically synthesized and bio assayed to show ACE inhibitory activity, as well as good stability in a simulated GI digestion (Yu et al., 2011).

Research aimed to identify new and existing biological activities of hen egg components will help to define new methods to further improve the value of eggs. Egg white protein powder (EWPP) is a novel egg-derived product that is being increasingly applied in the food processing industry because of its long shelf life. EWPP has been hydrolyzed by three different proteases and the enzymatic hydrolysates sequentially fractionated by ultrafiltration membranes.

Among the enzymes that were used alcalase can be considered the best enzyme for the preparation of antioxidant peptides derived from egg white protein (Lin et al., 2011).

The effect of cooking methods and GI digestion on the antioxidant activity of peptides derived from avian egg have been studied. The results suggest that fresh egg yolk has higher antioxidant activity than fresh egg white and whole eggs. Cooking of eggs reduced the antioxidant activity whereas simulated GI digestion increased it.

Boiled egg white hydrolysate showed the highest activity and a total of 63 peptides have been identified, which indicates the formation of novel antioxidant peptides. This results suggest the potential role of eggs as a dietary source of antioxidants (Remanan and Yu, 2014).

Three ovomucin hydrolysates have been prepared and desalted and only the desalted alcalase hydrolysate increased the proportion of low-MW peptides which showed anti-inflammatory activity. The showed biological activity was comparable to anti-inflammatory activity in dermal

fibroblasts. The anti-inflammatory activity of low-MW peptides was regulated through the inhibition of tumour necrosis-mediated nuclear factor κB pathway. This class of peptides may have potential applications for maintenance of dermal health and treatment of skin diseases (Sun et al., 2016).

Meat

eat and meat products are traditionally associated with increased risk of cancer, obesity, and other diseases, ignoring the role fact meat plays in human health. BP derived from meat products have the potential for incorporation into functional foods and nutraceuticals.

Meat and fish derived peptides have been shown to exhibit antihypertensive effects in vivo, along with antioxidant capabilities and other bioactivities such as antimicrobial and anti- proliferative activities in vitro (Ryan et al., 2011; Lafarga and Hayes, 2014; Mora et al., 2014; Liu et al., 2016; Ryder et al., 2016). The potential benefits of these compounds to human health has been recently reviewed.

Table 1: Some examples of BP from bovine milk proteins

Source	Peptides AA sequence	Bioactivity	Reference
Lactobacillus rhamnosus + digestion with pepsin	*Asp-Lys-Ile-His-Pro-Phe,Tyr-Gln-Glu-Pro-Val-Leu*	*ACE inhibitory*	(Mohanty *et al.*, 2016)
Lactobacillus helveticus	*Val-Pro-Pro, Ile-Pro-Pro*	*ACE inhibitory*	(Mohanty *et al.*, 2016)
Lactobacillus GG enzymes + pepsin and trypsin	*Tyr-Pro-Phe-Pro, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Thr-Thr-Met-Pro-Leu-Trp*	*Opioid, ACE inhibitory, immune-stimulatory*	(Mohanty *et al.*, 2016)
Lactobacillus delbrueckii subsp., bulgaricus IFO13953	*Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met*	*Antioxidative*	(Mohanty *et al.*, 2016)
Kluyveromyces marxianus var.	*Tyr-Leu-Leu-Phe*	*ACE inhibitory*	(Mohanty *et al.*, 2016)
β-Casein derived peptides	*Lys-Val-Leu-Pro-Val-P(Glu)*	*ACE inhibitory*	(Mohanty *et al.*, 2016)

(Mohanty *et al.*, 2016)

Exogenous peptides

Marine organisms (Ngo et al., 2012) are an important source for BP that have been employed for the treatment of various diseases (Kang et al., 2015; Manikkam et al., 2016). Chemical diversity is the result of the highly dynamic marine environment, and it represents an unlimited resource of new active substances with potential use as bioactive products. Marine organisms such as fish,

shellfish mollusks, marine processing waste, and crustaceans are abundant sources of a myriad of structurally diverse bioactive organic compounds (Aneiros and Garateix, 2004; Lee et al., 2012; Rustad and Hayes, 2012; Cheung et al., 2015; Jo et al., 2017).

The structures of some short peptides of marine origin, as well as the corresponding activity are represented. A well-documented evidence of their potential for human health (Fan et al., 2014; Ngo et al., 2012) which includes activities as antihypertensive (Kim et al., 2012), antioxidant (Ngo and Kim, 2013), antimicrobial (Kang et al., 2015; Falanga et al., 2016), anticoagulant, anti-diabetic (Manikkam et al., 2016), anticancer, immunostimulatory, calcium-binding, hypocholesteremic and appetite suppression has incentivized the interest of these compounds as functional food ingredients (Harnedy and Fitzgerald, 2012).

BP isolated and identified from crustaceans, regulate a large number of physiological functions, including colour change, heart activity, exoskeletal and visceral muscles, metabolic function, development, metamorphosis, and reproduction. Proteins derived from these marine organisms represent a unique source of proteins that can be used as raw materials for the generation of biofunctional peptides (Kim et al., 2012; Lee et al., 2012; Kim and Kim, 2013; Ngo and Kim, 2013; Kang et al., 2015; Falanga et al., 2016).

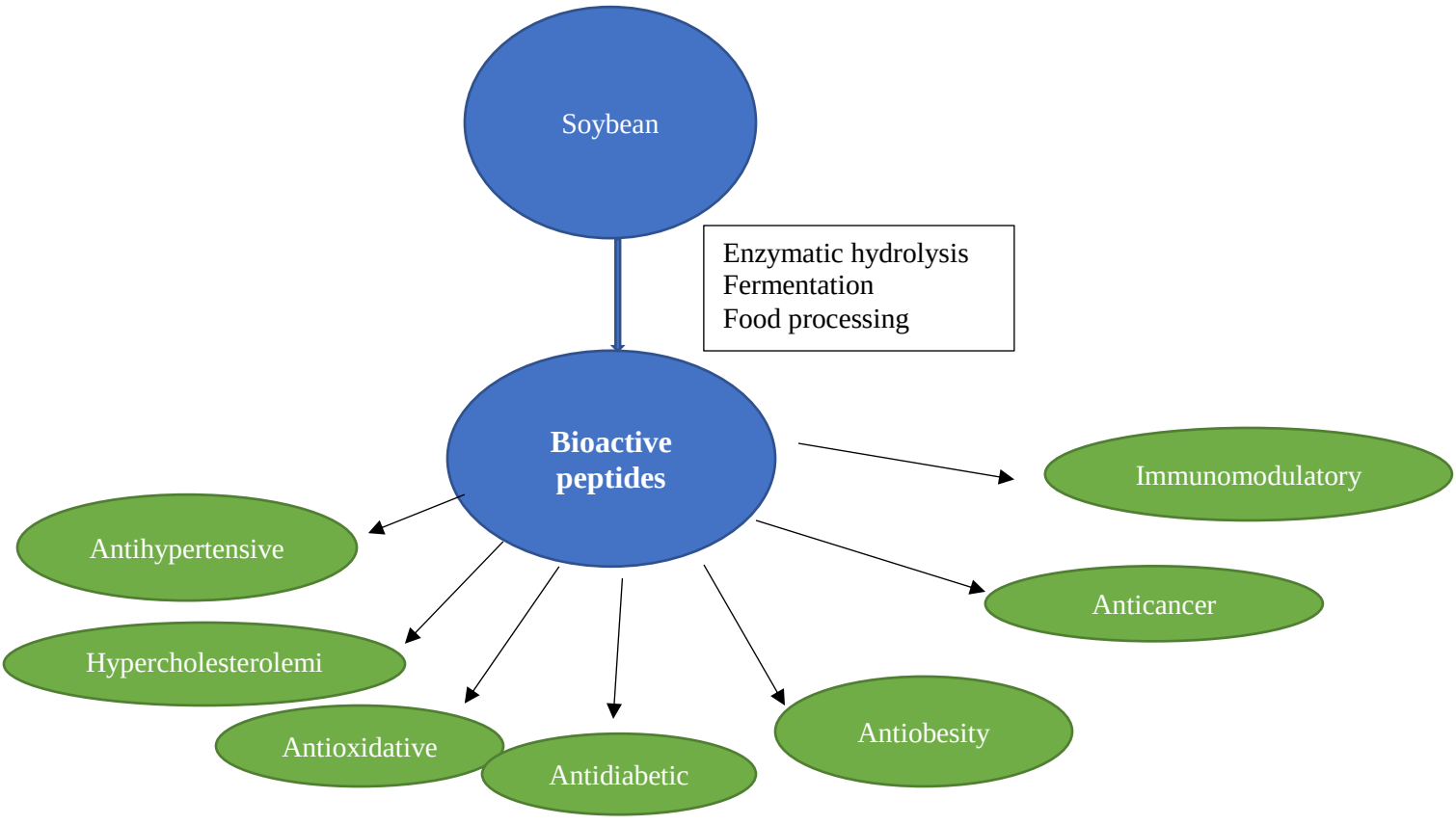
Bioactive peptides mechanism of action

Cardiovascular disease (CVD) has been recognized as the biggest cause of death worldwide. The renine-angiotensin system regulates blood pressure and fluid balance and plays an important role in the physiology of CVDs. Angiotensin converting enzyme (ACE), is a nonspecific dipeptidyl carboxy peptidase, converts the inactive decapeptide angiotensin I by cleaving dipeptide from the C-terminus into the potent vasoconstricting octapeptide angiotensin II in the renin-angiotensin system (RAS). This potent vasoconstrictor is also involved in the release of a sodium-retaining steroid, aldosterone, from the adrenal cortex, which has a tendency to increase blood pressure [Natesh R et al.2003]. ACE is widely distributed in mammalian tissues, predominantly as a membrane-bound ectoenzyme in vascular endothelial cells and also in several other cell types including absorptive epithelial, neuroepithelial, and male germi-nal cells (Sibony M et al.1993). ACE is a multifunctional enzyme that also catalyses the degradation of bradykinin, a blood pressure lowering nonapep-tide in the kallikrein–kinin system (Cat AN et al.2011) Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. ACE-inhibitory peptides block the first step in the renin-angiotensin system and interrupt the negative feedback effects of angiotensin II. ACE-inhibitory peptides, derived from dif-ferent plant and animal sources, when compared with chemosyn-thetic drugs, can be used as potent alternatives of synthetic drugs because of the increasing interest for safe and economical.

Oxidation reactions within the body during respiration in aer-obic organisms; particularly vertebrates and humans can produce free radicals (Evans JL et al.2003), as well as air pollutants and tobacco oxidants can be absorbed to blood circulation and exert adverse effects. In addition, UV radiation can stimulate the generation of a variety of oxidants (Hruza LL et al.1993). Oxidative damage plays a significantly patho-logical role in human diseases like cancer, emphysema, cirrhosis, atherosclerosis, and arthritis. When the mechanism of antioxi-dant protection becomes unbalanced by factors such as aging, deterioration of physiological functions may occur, resulting in diseases and accelerating aging. Antioxidant food supplements or bioactive peptides may be used to help the human body and ani-mals to reduce the oxidative damage (Young Jh et al 2000). Proteins and peptides can inhibit lipid oxidation through multiple

pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroper-oxides, and contribute to the endogenous antioxidant capacity of foods. Antioxidant activity of protein can be increased by their hydrolysis; peptides have substantially higher antioxidant activity than intact proteins (Elias Rj et al.2008). Dyslipidemia, characterized by the presence of one or more than one abnormal serum lipid concentration (total cholesterol-TC, LDL-C, triglycerides and HDL-C), is a prime risk factor for cardiovascular diseases (CVD). Atherosclerosis is a vascular chronic inflammation in the arterial wall that can lead to clinical manifestations including myocardial infarction, peripheral arterial disease and stroke. The mechanisms considered responsible for the hypocholesterolaemic activity of soy foods and its bioactive peptide involve stimulation of the secretion of bile acids, changes for cholesterol metabolism in the liver, hormonal effects and regulation of cholesterol receptors Anthony M et al.1997).

Figure:1 Biofunctional Soy Peptides (Singh et al. (2014).



Production of bioactive peptides

Bioactive peptides can be produced by enzymatic hydrolysis using proteolytic enzymes of digestive system or microbial origin, microbial fermentation and food processing (Fig. 1).

1.1-Enzymatic hydrolysis

Using single or multiple specific or nonspecific proteases, is the most common way for bioactive peptide production because it require shorter time to obtain a similar degree of hydrolysis as well as better control of the hydrolysis to obtain more consistent molecular weight profiles and peptide composition. These pro-cesses are especially used in food and pharmaceutical industries by several enzymes such as pepsin, bromelain, trypsin, chymotrypsin and papain under their respective optimal pH and temperature (Agyei D et al.2011). These enzymes are also used in combination to release more effective and stable bioactive peptides (Ayun T et al.2001) In addition sev-eral enzymes of plant origin, papain and pronase can be used for protein hydrolysis of soy flour and wheat flour (Frank F et al.2000). Soy protein hydrolysates have enzymatically prepared by several commercially available proteases, alcalase, flavourzyme, trypsin, papain, protease and peptidase (Kong XZ et al,2008). Many bioactive peptides, like biogenic, opi-oid, immunomodulating, mineral binding, antihypertensive and antimicrobial, can be produced by enzymatic hydrolysis of food materials such as milk, animal and fish meat, maize, wheat, soy-beans and egg (Yamamoto N et al.2003).

1.2-Food processing

Structural and chemical changes that occur during the food processing of protein may also lead to release of bioactive peptides(Korhonen H et.1998). In vivo digestion trials have shown that bioactive peptides can be formed during gastrointestinal digestion. N-methylation of peptides has been successfully used in peptide-based drug design to improve the metabolic stability because peptides and peptidomimetics having poor oral bioavailability(Gao J et al.2001). In vitro digestibility of three bioactive peptides from -lactoglobulin has been studied and found that when hydrolyzed with pepsin, - Lg f142–148 remained intact, whereas -Lg f15–20 and f102–105 were weakly hydrolysed (31%) with chymotrypsin, only -Lg f142–148 was strongly hydrolysed (99.8%) (Roufik S, et al.2006).

1.3-Fermentation

Fermentation is efficient way to produce bioactive peptides and food grade hydrolyzed proteins. Lactic acid bacteria (LAB), a large group of beneficial bacteria widespread in nature and are also found in our digestive systems, are generally used for bioactive peptides production. They are best known for their role in the preparation of fermented products, not only because of their physiological significance, but also because of their technological importance in texture and flavor development(Savijoki K et al.2006). The proteolytic system of lactic acid bacteria e.g.

Lactococcus (L.) lactis, Lactobacil-lus (Lb.) helveticus and Lactobacillus delbrueckii ssp. bulgaricus, have proteinases, broad specificity and are capable of releasing a large number of different oligopeptides (4–8 amino acid), oligopeptide transport system, main route for nitrogen entry into the cell and peptidases, located intracellular required for complete degradation of accumulated peptides (Christensen JE et al.1999) Lactic acid bacteria, possessing -galactosidase enzyme, can hydrolyze the soy oligosaccharides (sucrose, raffinose, and stachyose) during fermentation and reduce its beany flavor and flatulence (Dhananjay S,et al.2006) During fermentation of soy milk, proteins are degraded into simpler form like oligopep-tides, di-peptides and tri-peptides and serve as a good source of bioactive peptides. Comparatively microbial fermentation is the cheapest process instead of enzymatic hydrolysis for bioactive pep-tide production because microorganisms are a cheap source of proteases and recognized as safe. Bacterial cultivation costs are relatively low as they require minimal nutrition requirements and short time of maturation, moreover proteases of LABs are expressed on the cell membrane making purification protocols relatively easy and cheap (Agyei D, et al.2011).

Isolation, purification and characterization

High performance liquid chromatography (HPLC) is the most widely used technique for separation, identification, and purification of bioactive peptides. Sodium dodecyl sulfate (SDS)-electrophoresis and ultracentrifugation are alternative methods used for structural characterization and amino acid composi-tion of peptides. An ultrafiltration membrane system can be used to separate the peptides that have the desired molecu-lar weights and functional properties. Ultrafiltration membranes was used for screened bioactivities of soy protein hydrolysate and found increases biological activity of the recovered peptides (Rho SJ,et al.2009). Recently, electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI) and mass spectrometry (MS) have appeared as an important tool for protein identification and char-acterization. Liquid chromatography followed by tandem mass spectrometry detection (LC–MS/MS) is commonly used to iden-tify peptide sequences. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometric analysis is use-ful for generating peptide profiles of protein hydrolysates. Mass Spectrometry has allowed the accurate determination of molecu-lar mass and protein sequences as well as identification of protein degradation products and the study of protein conformations (Contreras M et al.2008).Membrane ultrafiltration and size-exclusion chromatography can be used to concentrate peptides of specified molecular weight ranges. In addition, reverse-phase HPLC can be used to fractionate peptides based on their hydrophobic properties especially when studying the structural functional properties of peptides (Pownall TL, et al.2010).

tripeptide (Ile-Gln-Asn) purified and identified from black soybean hydrolysate using chromatographic and protein sequencer, having an IC50 value of 0.014 mg protein/ml (Kim HJ et al.2007). Antioxidant peptides (Ala-Asp-Ala-Phe, 423.23 Da) was purified and identified from

wal-nut using RP-HPLC-ESI-MS, which identified for the first time from walnut protein hydrolysates. Angiotensin I-converting enzyme (ACE) inhibitory tripeptide (Gly-Pro-Pro), having IC50 value of 6.25 lg protein/ml, was also purified and identified from buckwheat protein by protein sequencing system and electrospray-LC–mass spectrometry,(Ma MS et al.2006). Seber and coworker developed a scalable method utilizes the sequential application of anion-exchange chro-matography, ultrafiltration, and reversed-phase chromatography to generates lunasin preparations of 99% purity with a yield of 442 mg/kg defatted soy flour. Mass spectrometry of the purified lunasin revealed that the peptide is 44 amino acids (Seber LE et al.2012)

Table:2 -Techniques used for isolation and identification of bioactive peptides

Techniques	Application	Identification of Bioactive peptides	Reference
Size exclusion chromotography	Used for fractionation of protein	Antioxidant peptide from enzymatic hydrolysis of sardinella	Bougatef A et al.2010.
Reversed phase High performance chromatography(RP-HPLC)	Separation of peptide from protein hydrolysate	ACE inhibitory peptides from fermented soybean extract.	Bougatef A et al.2010
Liquid chromatography-mass spectrometry(LCMS)	Physical separation of peptides and mass analysis	Hypercholesteremic peptide from soy protein hydrolysates	Gu Y et al.2013
Electrospray ionization-mass spectrometry (ESI-MS) and Tandem mass spectrometry (MS/MS)	Determination of molecular masses and amino acids sequences of the purified peptides	ACE inhibitory peptide (Gly-Pro-Pro) from buckwheat Antioxidant peptides (Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val) from grass carp muscle hydrolysates	Bougatef A et al.2010.
Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)	For generating peptide profiles of protein hydrolysates or semipurified fractions	ACE-inhibitory peptide (Val-Leu-Ile-Val-Pro) from soy protein	Mallikarjun Gouda KG et al.2006.

Literature of review

The soybean or soya bean (Glycine max) is a legume species native to East Asia, which is Hugely cultivated for its edible seed.

The plant has been classified under oil producing plant for its edible seed rather than for its pulse by the Food and Agricultural Organization (FAO). Soybean is an annual legume categorized under Fabaceae family.

Soybean is one of the most essential bean among all in the world which provides vegetable protein for millions of human and ingredients for thousands of chemical products. It is most nutritious and easily digested food of the bean family.
The soybean is considered as one of the richest and cheapest sources of protein. It is a staple in the diet of humans and animals in different corners of world today. The seed contains 17 percent oil and 63 percent meal, 50 percent of which is protein.

Soybean is aa good source of protein for diabetics as it contains no starch. Globally, the most valuable feed grain legume is soybean (Glycine max), with a total production of 216,144,262 tonnes and harvested area of 94,899,216 hectors (Faostat, 2009). Today, the world‘s top most producers of soybean are USA, Brazil, Argentina, India and China. Approximately 85 percent of the world‘s soybeans are processed, or "crushed," annually for the production of soybean meal and oil and 98 percent of the soybean meal that is crushed is further processed for preparation of animal feed. About 95 percent is consumed as edible oil; the rest is used for industrial products such as in the production of fatty acids, soaps and biodiesel. Soybean is an agricultural crop of great importance as many important proteins and nonproteinaceous compounds have been isolated. Soybean (Glycine max) seeds are known to contain different proteins which have anti nutritional and/or toxic effects, such as soybean agglutinin (an N-acetylgalactosamine-specific lectin).

Soybean lectin isolated from Glycine max is a carbohydrate binding protein highly specific to terminal non reducing N-acetyl-D-galactosamine but less to D-galactose. Presence of galactose during biofilm formation had various effects in the presence or absence of SBL.(Julieta Pérez-Giménez, et al. 2009). Soybean lectin has a vital role in the initial recognition of Rhizobium japonicumby the plant, which leads to a strain-specific, nitrogen-fixing symbiosis (Bohlool& Schmidt, 1974). Supplementation of soybean lectin (SBL) in diets resulted in a decrease in the activity of trypsin while protein levels and amylase activity increased in the pancreatic juice

(Hemalatha, C., 2011). Inhibitory effects of soybean proteinisolate (SPI) and soybean lectin on the intestinal absorption of nonheme iron were investigated by in vivo studies in rats. The soybean lectin has inhibitory effect on iron absorption (Sanae Hisayasa et al.). Soybean seeds from different cultivars have dissimilar protein products. The trypsin inhibitors of Bowman-Birk type is produced by yellow soybean (Losso JN., 2008). But the Chinese dull black soybean produces Kunitz type trypsin inhibitor (Lin. P,et al. 2008). The lectin of little black soybean was stable only up to 40 ºC for 20 min, rather the small glossy black soybean lectin was stable up to 70 ºC for 30 min. SBL isolated by the gel filtration chromatography gave a single peak but when subjected to electrophoresis in native conditions, moved as a single band and showed a native molecular mass of 110 kDa and under denaturing conditions the lectin gave a single band at a position of 30 kDa.

Soy bean nutritional value

Soybeans have a good source of protein, lipid, and other nutrients. Soy protein products can be good substitutes for animal products because, unlike some other beans, soy offers a "complete" protein profile. Soybeans contain all the essential amino acids (except methionine) (Synder HE et al.1987),

which must be supplied in the diet because they cannot be synthesized by the human body. Soy protein products can replace animal-based foods-which also have complete proteins but tend to contain more fat, especially saturated fat-without requiring major adjustments elsewhere in the diet. Proteins and lipids, some vitamins and minerals, are the major nutritionally important components of soybeans. Although carbohydrates are major constituents quantitatively, they play a minor nutritional role. This is mainly because soybeans are consumed more for their protein content and value, than for their carbohydrate contribution to the human diet. Also, structural carbohydrates are the major fraction of SB carbohydrates. Cereals, which tend to be higher in carbohydrate content than SB, are the major contributors of energy in human diets.Nevertheless, SB protein contributes about 45% to the energy in a meal in which it is the major component.Whole soy foods are also good sources of dietary fibre, Bvitamins, calcium, and omega-3 essential fatty acids, all important food components.

The Protein Content of Soybean Products.

Soy bean protein is particularly essential because it have an amino acid composition that complements that of cereals. Soybeans are limiting in the sulphurcontaining amino acids for most animal species, including humans, but contain sufficient lysine to overcome the lysine deficiency of cereals (Potter NH et al.1995). A soybean-rice combination is complementary for lysine and methionine and may help to explain the successful use of soy protein products such as soybean curd in the rice eating cultures of Asia. The amount of protein in soybeans, 38-44%, is larger than the protein content of other legumes, 20-30%, and much larger than that of cereals, 8-15% (Synder HE et al.1987).

Soybean Lipids and Micronutrient Profiles

Soy bean oil gives calories, the essential fatty acids and vitamins A and E, but contributes insignificant amounts of Vitamins D and K(Bates RP et al.1975). It has one of the highest iodine values of oilseeds (a value of 134), which is similar to that of sunflower oil; peanut butter, maize and palm oils have iodine values of 101, 127 and 51, respectively, indicating lower unsaturation compared to SB oil [26]. In SB oil, the fatty acids linoleic, oleic, palmitic and linolenic, make up 54, 24, 12 and 8%, respectivel (Potter NH et al.1995).) Due to the considerable unsaturation (unsaturated fatty acids are >85% of total fatty acids) of SB lipids, which tends to lower serum cholesterol,soybean consumption may be helpful. Soy oil can serve as a good source of oleic and the essential fatty acid (EFA) linoleic acid, with even the partially hydrogenated soy oil containing 25% linoleic and 3% linolenic acid, ((Potter NH et al.1995).

Soybean Carbohydrates

The carbohydrates of soybeans, containing little starch and hexose, are largely polysaccharides with some oligosaccharides. Carbohydrates make up approximately 35% of the SB. Approximately 50% of SB carbohydrates are nonstructural in nature and include: low molecular weight sugars, oligosaccharides and small amounts of starch. The other half comprises polysaccharides that include considerable amounts of pectic polysaccharides. The small amounts

of free galactose, glucose, fructose and sucrose make up the low molecular weight sugars. Galacto-oligosaccharides (raffinose, stachyose and verbascose) comprise approximately 5% of the SB dry matter, while starch represents less than 1% (Karr-Lilienthal LK et al.2005)

Soybean Minerals.

Dry soy bean has an ash content of about 5%, which is quite considerable. The major forms of minerals in SB are sulphates, phosphates and carbonates. Potassium is found in the SB in the highest concentration, followed by phosphorus, magnesium, sulphur, calcium, chloride and sodium in that order [31]. Minor minerals include silicon, iron, zinc, manganese, copper, molybdenum, fluoride, chromium, selenium, cobalt, cadmium, lead, arsenic, mercury, and iodine. They range from 0.01-140 ppm. Like other components, minerals in SB are influenced by variety, growing location and season, (O'Dell BL et al.1979).

Goitrogenic and Estrogenic Substances in Soybeans.

The isoflavone glucosides genistein, and glycitein-O-β-glucoside are the major soybean goitrogenic compounds. Other isoflavones, namely, 6,7,4'-trihydroxyflavone, coumesterol, are also found in soybeans in insignificant amounts. Diethylstilbesterol is 105 times more active than genistein, while daidzein is three-fourths as active as genistein, and so the estrogenic activity of soy isoflavones is usually thought to be minimal, (Bickoff EM et al.1962) The content of iodine in soybeans is insignificant, although it was shown that greater thyroid hypertrophy occurred when rats were fed raw full-fat soy flour than when they were fed lower iodine, toasted and defatted soy flour or SPI (Block RJ et al.1961).

Soybean Vitamins

The water-soluble vitamins of the Soy beans mainly include thiamine, riboflavin, niacin, pantothenic acid and folic acid. Vitamin C is negligible in the mature beans, but is present in measurable quantity in immature and germinated beans,(Bates RP et 1975).The main oilsoluble vitamins include A (retinol) and E (tocopherol). The Vitamin D and K content is negligible. Vitamin A exists as the pro-vitamin β-carotene. Like Vitamin C, its content is negligible in the mature bean, but is measurable in immature and germinated seeds, (Bates RP et 1975).

Soybean Omega-3 Fatty Acids

This family of fatty acids takes its name from the location of the double bond on the third carbon atom from the methyl (CH3) end of the fatty acid molecule (chain), known as the omega end. In soybeans, these fatty acids include alpha-linolenic acid (ALA) and linolenic acid (18:3, n-3). However, unlike other oil seeds used for food preparation, SB contains more omega-6 fatty acids than most other omega-3 oil seeds. This validates the classification of its oil as both an omega-3 and omega-6 commodity (Covington MB et al.2009).

Table 3: Composition (dry solids-g/100 g) of various foods

Food	Protein	Lipids	Carbohydrates	Moisture
Soybean	45	25	15	7
Peanuts	25	50	20	9
Pea	23	2	70	12
Field bean	30	2	60	12
Wheat (grain)	14	2	78	10
Maize	10	5	80	10
Sorghum	11	4	81	11
Cassava	3	0.8	90	63
Yam	9	0.7	84	73
Potato (tuber)	9	0	73	78

Cheftel JC et al.1985)

Table 4: Nutritional quality of proteins based on biological value evaluation and on amino acid composition

Protein	PER	BV	NPU	Limiting amino acid	Chemical score
Whole egg	3.8	87-97	91-94	None	100
Cow's milk	2.5	85-90	86	Sulphur amino acids	60
Beef muscle	3.2	76	71-76	Sulphur amino acids	80
Salmon	-	72	71	Tryptophan	75
Soybean	0.7-1.8	58-69	48-61	Sulphur amino acids	69
Peanut	1.7	56	43-54	Sulphur amino acids	70
Rice	1.9	75	70	Lysine	57
Maize	1.2	60	49-55	Lysine	55
Wheat	1.0	52	52	Lysine	57

(Synder HE et al.1987

Table 5: Protein content of various foods expressed as contribution (%) to energy from ach food

Value of food as a source of protein	Proportion of energy from protein (%)
Poor protein sources	
Cassava	3.3
Cooked bananas	4.0
Sweet potatoes (*Ipomoea batatas*)	4.4
Taros	6.8
Adequate protein sources	
Potatoes	7.6

Rice (home pounded)	8.0
Maize (wholemeal)	10.4
Millet (*Setaria italica*)	11.6
Millet (*Pennisetum glaucum*)	13.6
Sorghum (*Sorghum vulgare*)	11.6
Wheat flour (medium extraction)	13.2
Good protein sources	
Groundnuts (peanuts)	18.8
Cow's milk (3.5% fat)	21.6
Beans and peas	25.6
Beef (lean)	38.4
Cow's milk, skimmed	40.0
Soya bean	45.2
Fish, fatty	45.6
Fish, dried	61.6

(Passmore R et al.1986)

Problem statement

Bioactive peptides have a beneficial health effects like antihypertensive, antioxidative, antiobesity, immunomodulatory, antidiabetic, hypocholesterolemic and anticancer. So ,soybeans is one of the most abundant plant sources of dietary protein, contain 36–56% of protein. Soy milk, an aqueous extract of soybean, and its fermented product have great biological properties and are a good source of bioactive peptides.

Research Objectives

1. To obtain desirable chemical components of soybean
2. To extract, analyze and compare the peptides derived from different substrates.
3. To investigate the functional potential of the peptides for potential bioactivities.
4. To identify the potential peptides with bioactivities.
5. To study the application of bioactive peptides as food supplement and food packaging.

Research Methodology

❖ **Screening for proteolytic activity**

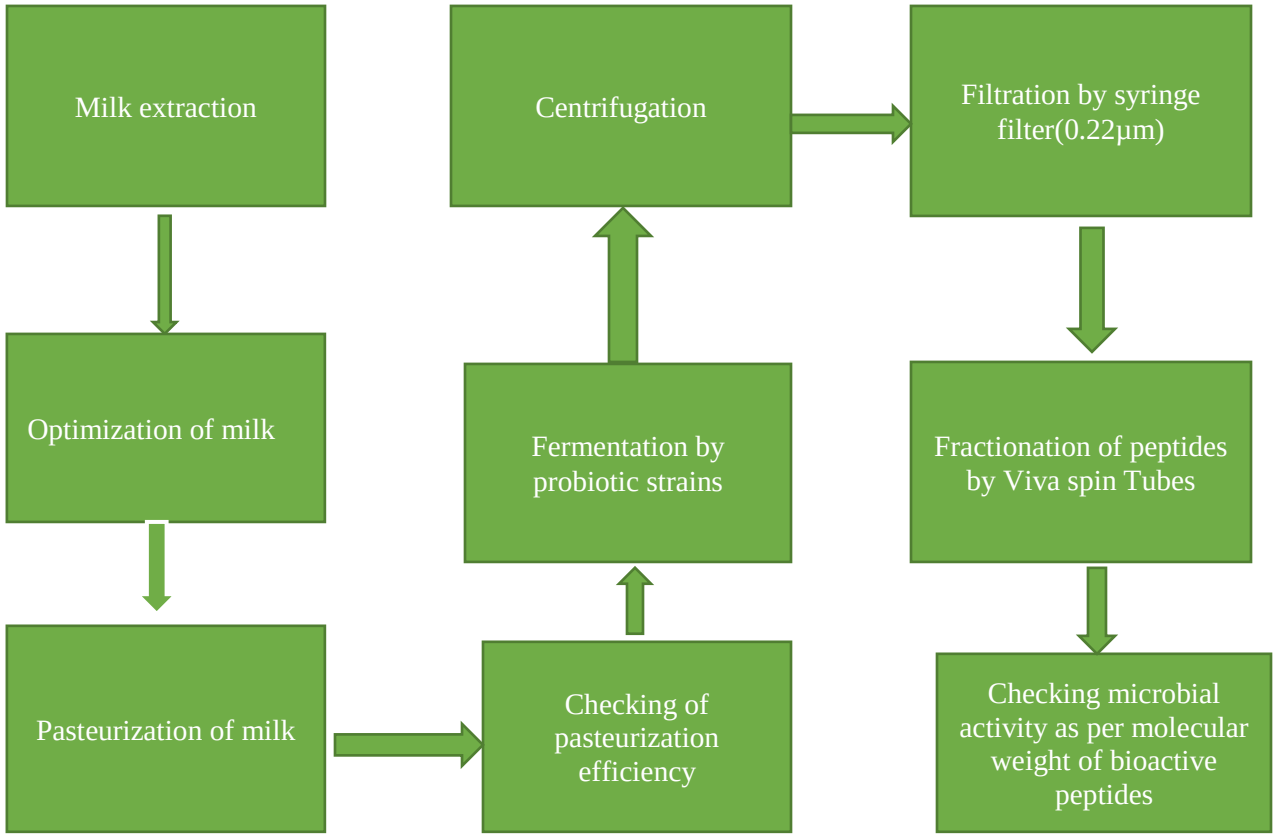
Proteolytic activity of different probiotic strains (Lactobacillus helveticus, Lactococcus lactis, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus brevis) will be checked by using spot inoculation and agar well diffusion method (Beganovic et al., 2012)

Skim milk agar plate will be used to check the proteolytic activity.

❖ **Extraction, analysis and comparison of peptides derived from different substrates**

Extraction of bioactive peptides will be done as per the method given by (Singh et al., 2015.)

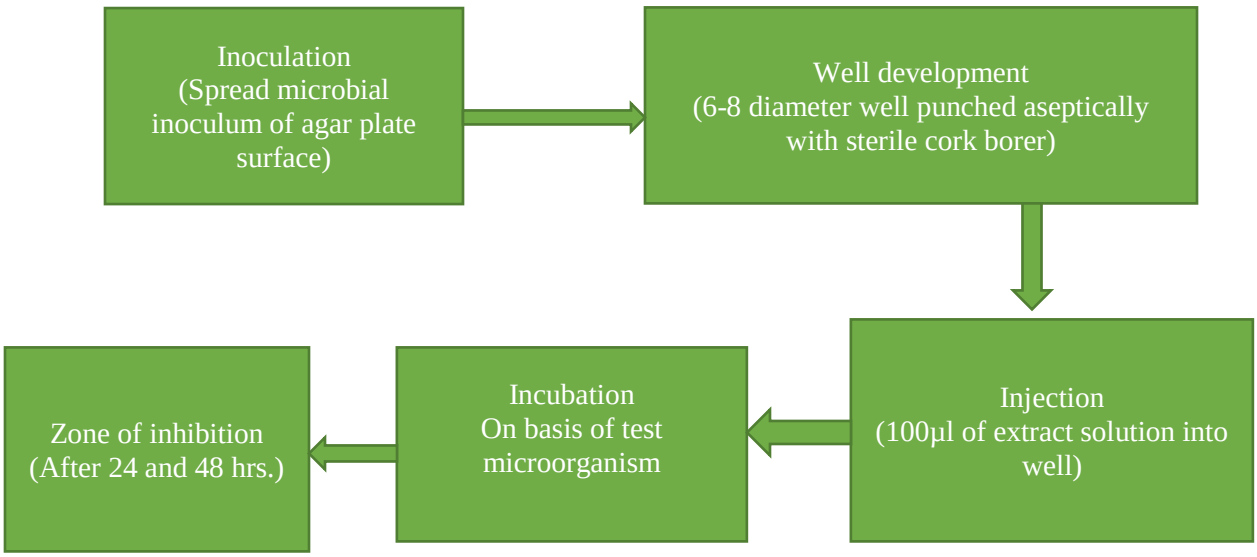
Figure:2 Extraction of bioactive peptides



❖ **Investigation of functional potential of peptides for potential bioactivities.**

Antimicrobial activity of extracted peptide will be checked by using Agar Well Diffusion Method (Singh et al., 2015)

Figure:3 Investigation of functional potential of peptides for potential bioactivities.



1. MATERIALS AND METHODS

1.1 Chemicals

All chemicals used for the present study were of analytical grade. The routine chemicals were procured from Hi media, SRL, Sigma and CDH and are as follows:

Lactobacillus MRS broth, Lactobacillus MRS agar (Hi media), Cysteine hydrochloric acid (SRL), L- leucine (Hi-Media), Peptone (Difco), o-phthaldialdehyde (Hi-Media), β-mercapto-ethanol (Sigma-aldrich), Sodium tetraborate (Hi-Media), Methanol (SRL), *n*-butanol (SRL), Acrylamide (Sigma), Bis-acrylamide (Sigma), Glycerol (SRL), Coomassie brilliant blue (SRL), Bromophenol blue (SRL), Glacial acetic acid (Qualigens), Potato dextrose agar (Hi-Media), D- Glucose (SRL), Sodium phosphate monobasic anhydrous (SRL), Sodium phosphate dibasic dihydrate (SRL), Hydrochloric acid (Rankem).

1.2 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.

1.3 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. Flaxseed was 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.

1.4 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.

1.5 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.

1.6. Optimization of water for flaxseed milk extraction

Soy bean was soaked in water at 45°C for 15 mins. Soaked flaxseeds were drained and the outer layer was removed (dehulling) mechanically followed by rinsing with running tap water. The soy bean was further grinded with water in different ratio (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7) in a high-speed blender. The SB slurry was indirectly heated in water bath at 85°C for 45 min and then filtered through double layer cheese muslin cloth to separate SB from residue and stored at refrigeration condition prior to use.

1.6.1. Physico-chemical analysis of flaxseed milk

Moisture content (AOAC, 2010)

Soy bean (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 flaxseed milk 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:

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

Where *TA*= titratable acidity

N= normality of NaOH

0.064= acid equivalent (Ranggana, 1977).

Alcohol test

Alcohol test was performed as per the method given by Kuthu *et al.,* 2013. 10ml of sample was taken and mixed with 5ml of alcohol and checked whether milk was clotted or not. This test was performed to check the heat stability of sample.

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.

Clot on boiling (COB)

COB test was performed as per the method given by Kuthu *et al.,* 2013. 5ml of sample was taken in test tube and heated for few minutes. This test was performed to check the coagulation in the milk sample.

TSS (total soluble solid)

Total solids were determined by subtracting the moisture %.

Protein test

Protein content was determined by using Kjeldahl method.

1.7. 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.

1.7.1. 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.

1.8. Preparation of flaxseed fermentation medium

Soy beans 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 (Varga, 2006).

1.9. Seed culture preparation for flaxseed 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 flaxseed milk and incubated at 37°C for fermentation. During fermentation milk was analyzed for change in pH, TSS, antioxidant and proteolytic activity.

1.10. Proteolysis assessment of fermented flaxseed milk 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).

1.10.1. 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.

1.10.2. 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.

1.10.3. 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.

1.10.4 Preparation of sample for proteolysis assessment

For the preparation of sample for proteolysis assessment, fermented flaxseed milk 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.

1.10.5 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.

1.11. Hydrolytic assessment of fermented flaxseed medium

Proteolysis of fermented sample was determined by SDS-PAGE electrophoresis. Fermented and non-fermented flaxseed milk 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.

1.11.1 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.

1.11.2. Materials

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

1.11.3. 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

1.11.4. 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

1.11.5. 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.

1.12. 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.

Expected outcomes

This research study is going to separate respective bioactive components such as lectin from soybean seeds and also differentiate those compounds from others of another seeds of plants. Finally, the extracted constituents are used for the prevention of chronic disorders such as cancer,hypertension and cure for diabetes and reduce the level of cholesterol and triglycerides in human body.

References

1- Kitts, D. D. and. Weiler, K. A. (2003). Bioactive proteins and peptides from food sources applications of bioprocesses used in isolation and recovery. Current Pharmaceutical Design, 9, 1309-1323.

2- FitzGerald R. and Murray B.A. (2006). Bioactive peptides and lactic fermentations. International Journal of Dairy Technology, 59, 118-125.

3- Mellander O. The physiological importance of the casein phosphopeptide calcium 665

4- salts. II. Per oral calcium dosage of infants. ActaSoc Med Uppsala 1950; 55:247–55.

5- Rutherfurd-Markwick KJ & Moughan PJ (2005) Bioactive peptides derived from food. JOAC Int 88, 955–966. Hernandez-Ledesma et al.2007.

6- Rizzello et al.2005.

7- Kearney, P.M.; Whelton, M.; Reynolds, K.; Muntner, P.; Whelton, P.K.; He, J. Global burden of hypertension. Lancet 2005, 365, 217–223.

8- Haque, E., Chand, R. and Kapila, S. (2009). Biofunctional properties of bioactive peptides of milk origin. Food Reviews International, 25, 1, 28-43.

9- Brulé, G., Roger, L., Fauquant, J. and Piot, M. 1982. Phosphopeptides from casein derived material. U.S. Patent 4, 358, 465.

10- Li, G.H.; Le, G.W.; Shi, Y.H.; Shrestha, S. Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. Nutr. Res. 2004, 24, 469–486.

11- Hayes, M.; Stanton, C.; Fitzgerald, G.F.; Ross, R.P. Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide functions. J. Biotechnol. 2007, 2, 435–449.

12- Katayama, K.; Tomatsu, M.; Fuchu, H.; Sugiyama, M.; Kawahara, S.; Yamauchi, K.; Kawamura, Y.; Muguruma, M. Purification and characterization of an angiotensin I converting enzyme inhibitory peptide derived from porcine troponin C. Anim. Sci. J. 2003, 74, 53–58. 86

13- Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., & Itoh, T. (1998) J. Dairy Sci. 81, 3131–3138 Faostat, (2009).Food and Agriculture Organization of the United Nations.

14- Julieta Pérez-Giménez, Elías J. Mongiardini, M. Julia Althabegoiti, Julieta Covelli, J. Ignacio Quelas, Silvina L. López-García, and Aníbal R. Lodeiro, (2009). Soybean Lectin

Enhances Biofilm Formation by Bradyrhizobium japonicum in the Absence of Plants, International Journal of Microbiology Volume 2009 Article ID 719367

15- Bohlool, B. B. and Schmidt, E. L., (1974).―Lectins: a possible basis for specificity in the Rhizobium—legume root nodule symbiosis,‖ Science, vol. 185: 269–271.

16- Hemalatha, C., Dhamotharan, R and Murugesan, S (2011). Effect Of Soya Bean Lectin On Streptozotocin Induced Diabetic Rats , Asian J. Exp. Biol. Scl. Vol 2(2): 231-236.

17- Pusztai,A. (1991). Plant lectins. Cambridge University Press, Cambridge.

18- Pusztai, A. (1993). Antinutritive effects of wheat-germ agglutinin and other Nacetylglucosamine-specific lectins.British Journal ofNutrition. 70: 313-321.

19- Peng Lin, Xiujuan Ye, and TB Ng(2008).Purification of melibiose-binding lectins from two cultivars of Chinese black Soybeans, Acta Biochim Biophys 1029-1038.

20- Grant, G., Bardocz, S., Brown, D. S.,Watt,W. B., Stewart, J. C. and Pusztai, A. (1990). Involvement of polyamines in pancreatic growthinduced by dietary soybean, lectin or trypsin inhibitors. Biochemical SocietyTransactions.18: 1009-1010.

21- Kennedy AR (1995).The evidence for soybean products as cancer preventive agents. J. Nutr. 125: 733S-743S.

22- Wong JH, Ng TB. (2005). A homotetr americanagglutinin withantiproliferative and mitogenic activities from haricot beans. J Chromatogr B Analyt Technol Biomed Life Sci 828: 130− 135.

23- de Mejia EG, Bradford T, Hasler C. (2003). The anticarcinogenic potential of soybean lectin and lunasin. Nutr Rev, 61: 239−246.

24- Agyei D, Danquah K. Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. Biotechnol Adv 2011;29:272–7.

25- Anthony M, Clarkson T, Bullock B, Wagner J. Soy protein versus soy phyto-estrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. Arterioscler Thromb Vasc Biol 1997;17(11): 2524–31.

26- Byun T, Kofod L, Blinkovsky A. Synergistic action of an X-prolyl dipeptidyl aminopeptidase and a non-specific aminopeptidase in protein hydrolysis. J Agric Food Chem 2001;49:2061–3.

27- Cat AN, Touyz RM. A new look at the renin–angiotensin system—focusing on the vascular system. Peptides 2011;32:2141–50.

28- Contreras M, Ivan L, Blanca H, Ramos M, Sidra R. Application of mass spec-trometry to the characterization and quantification of food-derived bioactive peptides. J AOAC Int 2008;91:981–94.
29- Dhananjay S, Kulkarni S, Kapanoor KG, Naganagouda VK, Veerappa HM. Reduction of flatus-inducing factors in soymilk by immobilized - galactosidase. Biotechnol Appl Biochem 2006;45:51–7.
30- Elias RJ, Kellerby SS, Decker EA. Antioxidant activity of proteins and peptides. Crit Rev Food Sci Nutr 2008;48:430–41.
31- Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Are oxidative stress-activated signaling pathways mediators of insulin resistance and -cell dysfunction. Diabetes 2003;52:1–8.
32- Christensen JE, Dudley EG, Pedersin JA, Steele JL. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie Leeuwenhoek 1999;76:217–46.
33- Franek F, Hohenwarter O, Katinger H. Plant protein hydrolysates: preparation of defined peptide fractions promoting growth and production in animal cells cultures. Biotechnol Prog 2000;6:688–92.
34- Gao J, Sudoh M, Aube J, Borchardt RT. Transport characteristics of peptides and peptidomimetics: I. N-methylated peptides as substrates for the oligopep-tide transporter and P-glycoprotein in the intestinal mucosa. J Pept Res 2001;57(4):316–29.
35- Hruza LL, Pentland AP. Mechanisms of UV-induced inflammation. J Invest Dermatol 1993;100:3–4.
36- Kim HJ, Bae IY, Ahn CW, Lee S, Lee HG. Purification and identification of adipo-genesis inhibitory peptide from black soybean protein hydrolysate. Peptides 2007;28:2098–103.
37- Kong XZ, Guo MM, Hua Y, Dong C, Zhang C. Enzymatic preparation of immunomodulating hydrolysates from soy proteins. Bioresour Technol 2008;99:8873–9.
38- Korhonen H, Pihlanto A, Rantama P, Tupasela T. Impact of processing on bioactive proteins and peptides. Trends Food Sci Technol 1998;9: 307–19.
39- Ma MS, Bae IY, Lee HG, Yang CB. Purification and identification of angiotensin I-converting enzyme inhibitory peptide from buckwheat (Fagopyrum escu-lentum Moench). Food Chem 2006;96:36–42.

40- Pownall TL, Udenigwe CC, Aluko RE. Amino acid composition and antioxi-dant properties of pea seed (Pisum sativum L.) enzymatic protein hydrolysate fractions. J Agric Food Chem 2010;58:4712–8.
41- Roblet C, Amiot J, Lavigne C, Marette A, Lessard M, Jean J, Ramassamy C, Moresoli C, Bazinet L. Screening of in vitro bioactivities of a soy protein hydrolysate separated by hollow fiber and spiral-wound ultrafiltration mem-branes. Food Res Int 2012;46:237–49.
42- Roufik S, Gauthier SF, Turgeon SL. In vitro digestibility of bioactive peptides derived from bovine beta-lactoglobulin. Int Dairy J 2006;16:294–302.
43- Savijoki K, Ingmer H, Varmanen P. Proteolytic systems of lactic acid bacteria. Appl Microbiol Biotechnol 2006;71:394–406.
44- Seber LE, Barnett BW, McConnell EJ, Hume SD, Cai J. Scalable purification and characterization of the anticancer lunasin peptide from soybean. PLoS ONE 2012;7(4):e35409.
45- Yamamoto N, Ejiri M, Mizuno S. Biogenic peptides and their potential use. Curr Pharm Des 2003;9:345–55.
46- Yang JH, Mau JL, Ko PT, Huang LC. Antioxidant properties of fermented soy-bean broth. Food Chem 2000;71:249–54.
47- Jang EH, Moon JS, Ko JH, Ahn CW, Lee HH, Shin JK, Park CS, Kang JH. Novel black soy peptides with antiobesity effects: activation of leptin-like signaling and AMP-activated protein kinase. Int J Obes 2008;32:1161–70.
48- Johnston JI, Franz VL. Renin-angiotensin system: a dual tissue and hormonal system for cardiovascular control. J Hypertens 1992;10:13–26.
49- proteins. Food Chem 2010;118:559–65. Bougatef A, Naima NA, Manni L, Ravallec R, Barkia A, Guillochon D, Nasri M. Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (Sardinella aurita) by-products
50- B.P. Singh et al. / Peptides 54 (2014) 171–179
51- Gu Y, Wu J. LC–MS/MS coupled with QSAR modeling in characterising of angiotensin I-converting enzyme inhibitory peptides from soybean proteins. Food Chem 2013;141:2682–90.

52- Mallikarjun Gouda KG, Gowda LR, Rao AG, Prakash V. Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (Glycine max). J Agric Food Chem 2006;54(13):4568–73.

53- Synder HE and TW Kwon Soybean Utilization. Van Nostrand Reinhold Co., New York, 1987.

54-

55- Potter NH and JH Hotchkiss Food Science, 5th edition. Chapman and Hall, New York, 1995: 362-363.

56-

57- Karr-Lilienthal LK, Kadzere CT, Grieshop CM and GC Fahley Jr Chemical and nutritional properties of soybean carbohydrates as related to nonruminants: A review. Livestock Production Science 2005; 97(1): 1-12.

58-

59- O'Dell BL Effect of soy protein on trace mineral availability. In: Wilcke HL, Hopkins DR and DH Waggle (Eds.). Academic Press, New York, 1979.

60-

61- Block RJ, Mandl RH, Howard HW, Bauer CD and DW Anderson The curative action of iodine on soybean goiter and the changes in the distribution of iodoamino acids in the serum and thyroid gland digests. Arch. Biochem. Biophys. 1961; 93: 15.

62-

63- Bates RP and RF Matthews Ascorbic acid and β-carotene in soybeans as influenced by maturity, sprouting, processing and storage. Proc. Fla. Hort. Soc. 1975; 88: 266-71.

64-

65- Covington MB Omega-3 fatty acids. Am. Family Physician J. 2004; 70: 133-140. Found at http://www.aafp.org.html. Accessed on 19th October, 2009.