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Antihypertensive Effect of Peptides from Sesame, Almond, and Pumpkin Seeds: In-silico and In-vivo Evaluation

Chelliah, R.[1,3], S. R. Ramakrishnan[2,3], U. Antony[3], S. H. Kim[1], I. Khan[1], C. N. Tango[1], P. N. Kounkeu[1], S. Wei[1], M. S. Hussain[1], E. B. M. Daliri[1], R. Momna[1], M. Y. Kwon[1], E. H. Lee[1], H. Y. Jo[1], S. B. Hwang[1], E. J. Park[1], H. J. Kim[1] and D. H. Oh[1*]

[1]Department of Food Science and Biotechnology, College of Agriculture and Life Science, Kangwon National University, Chuncheon 24341, Korea
[2]School of Food Science and Biotechnology, Kyungpook National University, Daegu 41566, Korea
[3]Centre for Food Technology, Department of Biotechnology, Anna University, Chennai 600025, India

***Corresponding author:** Oh, D. H. (E-mail: deoghwa@kangwon.ac.kr)

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A B S T R A C T

Plant proteins are important functional ingredients in many processed food products. In particular, globular proteins from various sources play an important role in many food products. In the current study, 11S globulin protein from white sesame seeds, amandin protein from almond seeds, and cucurbitin protein from pumpkin seeds were extracted by means of ammonium sulfate precipitation and purified by anion-exchange chromatography on a DEAE-Sephadex column (20 × 30 cm). Amandin protein of almond and cucurbitin protein of pumpkin seeds both belong to the 11S globulin family. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of protein samples treated with reducing agents revealed that the isolated 11S globulin from white sesame seeds was composed of an acidic (30 − 33 kDa) and basic (20 − 24 kDa) polypeptide, amandin from almond seeds was composed of an acidic (40 − 42 kDa) and basic (15 − 20 kDa) polypeptide, and cucurbitin from pumpkin seeds was composed of an acidic (35 − 42 kDa) and basic (20 − 25 kDa) polypeptide. The alpha amylase-inhibiting activities of the three proteins was determined. Cucurbitin of pumpkin seeds had a greater alpha amylase inhibitory effect (− 86%) than 11S globulin of sesame (82.6%) and amandin protein of almond seeds (76%). The anti-hypertensive effect of the three proteins was evaluated by a chrioallantoin membrane assay in chick embryos, which revealed that cucurbitin protein showed higher vasodilatation activity than the other two proteins.

Keywords: Alpha amylase, Amandin, Antihypertensive, Cucurbitin, Globular proteins

Introduction

Plant proteins are important functional ingredients in many processed food products. Particularly globular proteins from various sources play an important role in many food products, due their contribution to food texture (Hosseinian et al., 2016). These texture contributions come from the network structures created by the proteins. Gelation is one of the most important functional properties of the globular proteins used to modify food texture

(Scholten et al., 2014).

Seed storage proteins have been traditionally classified through the sequential extraction of crushed and defatted seeds by a series of aqueous and non-aqueous solvents (Scotter, 2011). The fraction extracted with water is defined as albumins, the fraction with dilute salt as globulins, the fraction with ethanol as prolamines, and the fraction with acid or alkali as glutelins. In cereal grains the main storage proteins are usually the alcohol soluble prolamines, whereas, in non-cereal grains the more nutritiously balanced salt soluble globulins predominate (Aloisi et al., 2016). Globulins can be subdivided into two distinct classes termed 7S and 11S on the basis of their sedimentation coefficient (Singh et al., 2015). Dicotyledonous plants have been found to favour the presence of the 11S globulin form (Kumar et al., 2017). 11S globulins are found to occur in the 300 kDa range whereas 7S globulins are generally less abundant and found to occur in the 180 kDa range.

The globulins storage proteins are classified into two broad groups, on the basis of their sedimentation coefficients: 7S Vicilin-type and 11S Legumin-type. The majority of the storage globulin proteins are soluble in dilute salt solution but insoluble in water. The two main reserve proteins of soybean that are used in the food industry are 7S β- conglycinin and 11S glycinin globulins. The 7S globulin is a trimeric glycoprotein (141 − 170 kDa) composed of three subunits, α (57 kDa), α' (58 kDa) and β (42 kDa), associated by hydrophobic interactions (Mutambuka, 2013). Sesame *(Sesamum indicum*) is the most ancient oil seed known and used by humans as a food source. Sesame oil is different from all other vegetable oils in many chemical, biological and physiological properties. These properties are due to the presence of endogenous unsaponifiable constituents viz, sesamol, sesamin, and sesamolin. Sesamin is reported to possess *in vivo* hypocholesterolemic activity and suppressive activity against chemically induced cancer (Bedigian, 2010).

Almond *(Prunus dulcis*), belongs to the Rosaceae family that also includes apples, pears, prunes, and raspberries. Almond is one of the most popular tree nuts on a worldwide basis and ranks number one in tree nut production (Sathe et al., 2002). They are typically used as snack foods and as ingredients in a variety of processed foods, especially in bakery and confectionery products (Moubarac et al., 2014). Extracts of whole almond seed, brown skin, shell, and green shell cover (hull) possess potent free radical-scavenging capacities (Jayasena, 2011). In addition, almonds, when used as snacks and in diets of hyperlipidemic subjects, significantly reduced coronary heart disease (Sweazea et al., 2014). A longterm supplementation of almond showed spontaneous nutrient modification of an individual's habitual diet that closely matched the recommendations to prevent cardiovascular and other chronic diseases (Sweazea et al., 2014).

Pumpkin (*Cucurbita maxima*) belongs to the family cucurbitaceae. Pumpkin seed proteins, beside their wide use as food ingredients, have pharmacological activities too such as antidiabetic (Park et al., 2015), antifungal (Abdel-Rahim et al., 2015), antibacterial and anti-inflammation activities (Perez Gutierrez et al., 2016). Pumpkin storage proteins are 2S albumins and 11S globulins (cucurbitin) localized in the protein bodies (Perez Gutierrez, 2016).

Hypertension is a growing undesired symptom that damages health and threatens mostly the developed societies. It is one among the major independent risk factors for atherosclerosis, stroke, myocardial infarction, and end stage renal disease. Angiotensin I-converting enzyme, adipeptidyl-dipeptidase, is the key enzyme that functions in the

rennin angiotensin system to increases blood pressure (Hermida et al., 2016). ACE increases blood pressure by both converting the inactive decapeptide Angiotensin I to the potent vasoconstrictor Angiotensin II and inactivating the vasodilator bradykinin. Inhibition of ACE is considered the first line of therapy for hypertension and atherosclerosis. ACE inhibitors have short peptides that bind tightly to the active site of ACE competing with Angiotensin I for occupancy (Duan et al., 2014).

ACE inhibitor peptides derived from daily dietary food proteins would be useful in the development of a novel functional food additive and present a healthier and natural alternative to ACE inhibitor drugs (Te Riet et al., 2015). The present study focus on 11S globulin protein from white sesame seeds, amandin protein from defatted sweet almond seeds and cucurbitin protein from defatted pumpkin seeds; to purify 11S globulin, amandin and cucurbitin protein by column chromatography; further to characterize 11S globulin, amandin and cucurbitin protein by SDS Page and in addition compare the alpha amylase inhibiting activity and anti-hypertensive activity of 11S globulin, amandin and cucurbitin whole protein.

Materials and Methods

Plant materials applied in the study

Commercial samples of white sesame, sweet almond and pumpkin seeds were obtained from local market (Chennai, South India, month-September 2017).

Extraction of fat from white sesame, almond and pumpkin seeds

Plant materials (sesame, sweet almond and pumpkin seeds) were dried at 45°C for 12 hours and grounded to fine powder using mechanical blender. And further defatted with hexane and cold acetone using a soxhlet apparatus for 16 h (1 : 10, w : v, flour to hexane and cold acetone solvent ratio). After extraction was completed the oil flask was dried in an air oven for three hours at 100 to 105°C. It was then cooled in a desiccator and weighed. Fat% = $(W_2 - W_1)/W \times 100$ (W-Weight of sample before defatting, W_1-Weight of empty flask, W_2-Weight of flask after defatting). Defatted almond flour was dried at room temperature overnight and used for protein extraction.

Extraction of amandin protein by ammonium sulphate precipitation

At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule, thus attracting protein into the solution and enhancing the solubility of protein. This is commonly known as salting-in. However, as the salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration implies that there is less and less water available to solubilize protein. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules. This phenomenon of protein precipitation in the presence of excess salt is known as salting-out. Inorganic salts are used for protein precipitation. Most commonly ammonium sulphate is used for precipitation. Advantages of using

ammonium sulphate for precipitation are at saturation, it is of sufficiently high molarity that it causes the precipitation of most proteins. It does not have a large heat of solution, allowing heat generated to be easily dissipated. Its saturated solution (4.04 M at 20°C) has a density (1.235 g cm^{-3}) that does not interfere with the sedimentation of most precipitated proteins by centrifugation. Its concentrated solutions are generally bacteriostatic. In solution it protects most proteins from denaturation.

Extraction of 11s globulin protein from defatted white sesame seeds

Sesame defatted flour was dissolved in phosphate buffer (20 mM, pH 7.5) containing 1 M sodium chloride (1 : 10, w/v, sesame flour to solvent ratio).It was kept for constant stirring at room temperature for 2 hr. The slurry was centrifuged at 19,800 × g for 1 hour at 4°C. The supernatant obtained after centrifugation was filtered through, Whatman no 1 filter paper. The filtered supernatant obtained was diluted with distilled water (1 : 5.5, v/v) and allowed to stand for 1 hour and centrifuged at 19,800 × g for 1 hour at 4°C. The water-insoluble pellet was re-dissolved in the extraction buffer and ammonium sulphate was added to 0 − 35% saturation. The solution was left to stand for 2 hours and subsequently centrifuged at 11,600 × g for 30 min at 4°C. The obtained pellet was re-dissolved in the extraction buffer, dialyzed extensively against distilled water. The obtained protein sample was freeze-dried. This fraction was the starting material for the purification of the sesame 11S globulin by column chromatography.

Extraction of amandin protein from defatted sweet almond seeds

Defatted almond flour was dissolved in Tris-HCl (50 mM, pH 7.5) containing 150 mM sodium chloride (1 : 10, w/v, defatted almond flour to solvent ratio). It was kept for constant stirring at room temperature for 2 hours. The slurry was centrifuged at 19,800 × g for 1 hour at 4°C. The supernatant obtained after centrifugation was filtered through Whatman no. 1 filter paper. The filtered supernatant obtained was subjected to salt fractionation of 0 − 40% (w/v) ammonium sulphate. The solution was left to stand overnight and subsequently centrifuged at 11,600 × g for 30 min at 4°C. The obtained pellet was re-dissolved in the extraction buffer, dialyzed extensively against distilled water for 24 hours. The obtained protein sample was freeze dried. The protein sample was further purified by ion-exchange column chromatography.

Extraction of cucurbitin protein from defatted pumpkin seeds

Defatted pumpkin seeds flour was dissolved in Tris-HCl (30 mM, pH 7.5) containing 1 M sodium chloride (1 : 10, w/v, defatted pumpkin flour to solvent ratio). It was kept for constant stirring at room temperature for 2 hr. The slurry was centrifuged at 19,800 × g for 1 hour at 4°C. The supernatant obtained after centrifugation was filtered through Whatman no. 1 filter paper. The filtered supernatant obtained was subjected to salt fractionation of 0 − 40% (w/v) ammonium sulphate. The solution was left to stand overnight and subsequently centrifuged at 11,600 × g for 30 min at 4°C. The obtained pellet was re-dissolved in the extraction buffer, dialyzed extensively against distilled

water for 24 hours. The obtained protein sample was freeze dried. The protein sample was further purified by ion-exchange column chromatography.

Purification of protein by ion exchange chromatography

An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix. It is possible to have both positively and negatively charged exchangers. Positively charged exchangers have negatively charged counter-ions (anions) available for exchange and are called anion exchangers. Negatively charged exchangers have positively charged counter-ions (cations) and are termed cation exchangers. The charge of a protein is determined by its pI and the buffer pH. If the pH is greater than the pI, the protein will have a negative charge. If the pH is less than the pI, then the protein will have a positive charge.

Gradient salt elution

Salt gradient elution strategy is that the weakest bound ions should be eluted first.

Purification of 11s globulin protein of white sesame seeds, amandin protein of almond seeds and cucurbitin protein of pumpkin seeds by DEAE- column chromatography

DEAE-Sephadex A-50 column was equilibrated with 0.05 M tris-HCl buffers (pH 7.5). The freeze dried sample was dissolved in 0.05 M tris-HCl buffer (pH 7.5). The sample solution was loaded to the equilibrated DEAE-Sephadex column. The protein sample was eluted from the column using 0.05 M Tris-HCl buffer (pH 7.5) containing different concentrations of NaCl. 0.05 M tris-HCl buffer (pH 7.5) containing 100, 200, 400, 600, and 1,000 mM NaCl was used for elution.

Protein estimation by bradford method

Total 200 μL of Bradford reagent was added to ELISA plate, BSA of different concentration was prepared (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg/mL), 10 μL of BSA sample was added to ELISA plate, incubated for 10 min at room temperature, blank is taken without protein sample. Absorbance was measured at 595 nm. Protein sample eluted by different concentrations of sodium chloride (NaCl) was taken (100, 200, 400, 600, and 1,000 mM), 10 μL of protein sample was added to ELISA plate, and Incubated for 10 min at room temperature, Blank is taken without protein sample. Absorbance was measured at 600 nm.

Characterization of 11s globulin protein of Sesame seeds, amandin protein of almond seeds and cucurbitin protein of pumpkin seeds using SDS-PAGE

The SDS-PAGE analysis was performed under reducing conditions. The following polypeptides were used as

low range molecular weight marker: phosphorylase b (102 kDa), bovine serum albumin (59 kDa), ovalbumin (42 kDa), carbonic anhydrase (28 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (16 kDa).

MALDI-TOF

MALDI is attached to a time of flight (TOF) analyzer which measures the time it takes for the molecules to travel a fixed distance. MALDI is a soft ionization technique in which a short laser pulse is used to ionize molecules. A protein or peptide sample is placed on a target plate and mixed with an appropriate matrix on the target plate. The mixture of sample and matrix crystallizes due to the vacuum environment and then is irradiated with a short laser pulse. The sample molecules and the matrix now enter gas phase. This leads to release of matrix, samples molecules, and ions from the target plate. The ions then accelerate in TOF analyzer when subjected to electric field. The ions travel in a strait and linear direction to the detector. The mass to charge ratio (m/z) of the sample ions is determined.

Alpha-amylase enzyme inhibition assay

Maltose, a reducing sugar, is detected by performing an oxidation reduction reaction. Due to the presence of a carbonyl group (C = O), maltose participates in an oxidation-reduction reaction with DNS (3, 5-dinitrosalicylic acid). The carbonyl group of maltose gets oxidized and changed into a carboxyl group and the 3, 5-dinitrosalicylic acid is reduced and changed into 3-amino, 5 nitrosalicylic acid (simply can be termed as reduced DNS). This reaction cause a change in color form yellow to red, depending on the concentration of maltose produced. Presence of alpha amylase inhibitor inhibits the maltose production from starch. The value of absorbance is measured on the “samples” and is interpreted as the amount of maltose produced, thus showing the extent of inhibition of amylase activity.

Antihypertensive assay

Chorioallantoin membrane assay

The allantois of the chick embryo appears at about 3.5 days of incubation as an invagination from the ventral wall of the endodermal hind gut. During the fourth day, it pushes out of the body of the embryo into the extraembryonic coelom. Its proximal portion lies parallel and just caudal to the yolk sac. When the distal portion grows clear of the embryo it becomes enlarged. The narrow proximal portion is known as the allantoic stalk, and the enlarged distal portion as the allantoic vesicle. Fluid accumulation distends the allantois such that its terminal portion resembles a balloon in embryos. The allantoic vesicle enlarges very rapidly from days 4 − 10 of incubation. In this process, the mesodermal layer of the allantois becomes fused with the adjacent mesodermal layer of the chorion to form the CAM (chorioallantoin membrane). A double layer of mesoderm is thus created: its chorionic component is somatic mesoderm and its allantoic component is splanchnic mesoderm. In this double layer an extremely rich vascular network develops which is connected to embryonic circulation by the allantoic arteries and veins. Chick eggs were obtained, Chick eggs were incubated in 37.5°C and 80% relative humidified environment. On the day 5 of incubation period, 5 ml of albumin was taken from the solid side of the eggs by a syringe allowing detachment of

the embryo from the eggshell. A shell piece of 2 − 3 cm in diameter was removed to open a window on the other side of the eggs, Normal development was verified and embryos with malformations or dead embryos were excluded. Whatman filter paper disc was placed over the chrioallantoin membrane and 200 ul of sample was applied to the disc. SpNO (Spermine Nonoate), an NO donor was used as positive control. Adrenaline was used as negative control, Images were taken using stereo microscope. Blood vessels diameter was measured using ImageJ software.

Results and Discussion

Psi blast based homology search

Glycinin protein of soybean is a rich source of bioactive peptides. In the current study, using PSI-BLAST program, searches for sequences similar to the query protein soybean glycinin was done. Homologs of soybean glycinin were found in 11S globulin protein of white sesame seeds, amandin protein of almond seeds and cucurbitin protein of pumpkin seeds. Food protein sources with bioactive peptides have been screened based on the homology of protein sequences analyzed using PSI BLAST Program.

The pairwise alignments showing the similarity between glycinin of soybean and 11S globulin of sesame seeds (Table 1). Natural alpha amylase and glucosidase inhibitors from the dietary plants can be used as an effective therapy for treating post prandial hyperglycemia. Recent studies focused on the search for more effective inhibitors of anti-diabetic compounds from natural materials (Jhong et al., 2014), such as polysaccharides from tea leaves (Sacan et al., 2017), hydrolysate from sardine muscle (Hayes et al., 2015) and isoflavones from soybean (Lee et al., 2015). Egg protein hydrolysates displayed anti-diabetic activity against glucosidase activity (Zambrowicz, 2015). Bioactive peptides from albumin showing α- glucosidase and α-amylase were identified. The inhibitory activities of peptides KLPGF, EVSGL, QITKPN, AEAGVD, EAGVD, NVLQPS, LEPINF, and ANEIIF from albumin were evaluated according to the method described by Kim (Yu et al., 2012) with some modification. The peptides KLPGF and NVLQPS showed significant inhibitory effects on α-glucosidase. Peptide KLPGF, and EAGVD exhibited significant inhibitory activity against the α-amylase. Peptide KLPGF was a potential anti-diabetic inhibitor (Jakubczyk et al., 2017).

In-silico analysis for identification of proteins with bioactive peptides in sesame

Glycinin protein of soybean which is a rich source of bioactive peptides was chosen as the query sequence. A homology search was performed against the genus Sesame, Almond and Pumpkin using PSIBLAST algorithm. The pairwise alignments of soybean glycinin with the corresponding homologues from sesame, almond and pumpkin were stored. Using a peptide library of antioxidant and anti-hypertensive peptides, positions of these bioactive peptides were identified in the 11 S globulin sequences from sesame, almond and pumpkin seed (Table 1). Defatted sesame meal contains more sugar and is generally utilized as animal feed has a great potential in combating the protein calories, functional properties of sesame protein concentrate (Serna-Saldivar, 2015).

Screening of antihypertensive and alpha amylase inhibiting peptides

Screening of biologically active peptides was done using constructed library of peptides (Table 1).

Table 1. Screening of antihypertensive and alpha amylase inhibiting peptides, alpha amylase inhibiting peptides

Isolated Proteins	PSI-BLAST algorithm (pairwise alignments of soybean glycinin with the corresponding homologues)
11S GLOBULIN (SESAME)	MVAFKFLLALSLSLLVSAAIAQTREPRLTQGQQCRFQRISGAQPSLRIQSEGGTTELWD ERQEQFQCAGIVAMRSTIRPNGLSLPNYHPSPRLVYIERGQGLISIVPGCAETYQVHRSQ RTMERTEASEQQDRGSVRDLHQKVHRLRQGDIVAIPSGAAHWCYNDGSEDLVAVSIN DVNHLSNQLDQKFRAFYLAGGVPRSGEQEQQARQTFHNIFRAFDAELLSEAFNVPQET IRRMQSEEEERGLIVMARERMTFVRPDEEEGEQEHRGRQLDNGLEETFCTMKFRTNVE SRREADIFSRQAGRVHVVDRNKLPILKYMDLSAEKGNLYSNALVSPDWSMTGHTIVY VTRGDAQVQVVDHNGQALMNDRVNQGEMFVVPQYYTSTARAGNNGFEWVAFKTT GSPMRSPLAGYTSVIRAMPLQVITNSYQISPNQAQALKMNRGSQSFLLSPGGRRS
PRUNIN (ALMOND)	MAKAFVFSLCLLLVFNGCLAARQSQLSPQNQCQLNQLQAREPDNRIQAEAGQIETWNF NQEDFQCAGVAASRITIQRNGLHLPSYSNAPQLIYIVQGRGVLGAVFSGCPETFEESQQ SSQQGRQQEQEQERQQQQQGEQGRQQGQQEQQQERQGRQQGRQQQEEGRQQEQQQ GQQGRPQQQQQFRQFDRHQKTRRIREGDVVAIPAGVAYWSYNDGDQELVAVNLFHV SSDHNQLDQNPRKFYLAGNPENEFNQQGQSQPRQQGEQGRPGQHQQPFGRPRQQEQQ GSGNNVFSGFNTQLLAQALNVNEETARNLQGQNDNRNQIIRVRGNLDFVQPPRGRQE REHEERQQEQLQQERQQQGGQLMANGLEETFCSLRLKENIGNPERADIFSPRAGRISTL NSHNLPILRFLRLSAERGFFYRNGIYSPHWNVNAHSVVYVIRGNARVQVVNENGDAIL DQEVQQGQLFIVPQNHGVIQQAGNQGFEYFAFKTEENAFINTLAGRTSFLRALPDEVL ANAYQISREQARQLKYNRQETIALSSSQQRRAVVCLLLLFNGCLASRQHIFGQNKEWQ LNQLEAREPDNHIQSEAGVTESWNPSDPQFQLAGVAVVRRTIEPNGLHLPSYVNAPQLI YIVRGRGVLGAVFPGCAETFEDSQPQQFQQQQQQQQFRPSRQEGGQGQQQFQGEDQQ DRHQKIRHIREGDIIALPAGVAYWSYNNGEQPLVAVSLLDLNNDQNQLDQVPRRFYLA GNPQDEFNPQQQGRQQQQQQQGQQGNGNNIFSGFDTQLLAQALNVNPETARNLQGQ DDNRNEIVRVQGQLDFVSPFSRSAGGRGDQERQQEEQQSQREREEKQREQEQQGGGG QDNGVEETFCSARLSQNIGDPSRADFYNPQGGRISVV NRNHLPILRYLRLSAEKGVLYNNAIYTPHWHTNANALVYAIRGNARVQVVNENGDPI LDDEVREGQLFLIPQNHAVITQASNEGFEYISFRTDENGFTNTLAGRTSVLRALPDEVL QNAFRISRQEARNLKYNRQESRLLSATSPPRGRLMSILGY
CUCURBITIN (PUMPKIN)	MARSSLFTFLCLAVFINGCLSQIEQQSPWEFQGSEVWQQHRYQSPRACRLENLRAQDP VRRAEAEAIFTEVWDQDNDEFQCAGVNMIRHTIRPKGLLLPGFSNAPKLIFVAQGFGIR GIAIPGCAETYQTDLRRSQSAGSAFKDQHQKIRPFREGDLLVVPAGVSHWMYNRGQSD LVLIVFADTRNVANQIDPYLRKFYLAGRPEQVERGVEEWERSSRKGSSGEKSGNIFSGF ADEFLEEAFQIDGGLVRKLKGEDDERDRIVQVDEDFEVLLPEKDEEERSRGRYIESESE SENGLEETICTLRLKQNIGRSVRADVFNPRGGRISTANYHTLPILRQVRLSAERGVLYSN AMVAPHYTVNSHSVMYATRGNARVQVVDNFGQSVFDGEVREGQVLMIPQNFVVIKR ASDRGFEWIAFKTNDNAITNLLAGRVSQMRMLPLGVLSNMYRISREEAQRLKYGQQE MRVLSPGRSQGRREF
Antihypertensive peptides Tripeptides	IKP, IKW, IKY, IRP, LKF, LKP, LKW, LRF, LRP, LRW, LRY, VRF and VRP
Alpha amylase inhibiting Peptides	KLPGF, EVSGL, QITKPN, AEAGVD

In vitro Studies

In vitro studies were carried out with sesame, almond and pumpkin seed. Sesame, almond and pumpkin seeds are rich source of proteins. The objective of the present study is to determine the bioactivity of 11s globulin protein in sesame, amandin protein in almonds, cucurbitin protein in pumpkin seeds (Table 2).

Table 2. Percentage of alpha amylase inhibition with different concentrations of 11s globulin protein of sesame seed, total protein and lipid content of sesame, almond and pumpkin

Concentration (mg/mL)	Alpha amylase inhibition (%)		
	11S globulin protein of sesame seed	Amandin protein of almond seeds	Cucurbitin protein of pumpkin seeds
0.1	63.7	58	70.8
0.2	64.5	60	74.8
0.3	66.3	63.4	75.10
0.4	69.3	65.5	75.3
0.5	70.4	68.4	76.18
0.6	70.1	70	76.3
0.7	71.4	70.9	77.3
0.8	71.9	72.8	78
0.9	74.7	75	80
1.0	82.9	78	86
Protein	7.14	6.46	3.76
Lipid (%)	59.4	58.9	51.2

Determination of fat percentage in sesame, almond and pumpkin seeds

The percentage of fat in white sesame seeds was found to be 59.4% when defatted with hexane using a soxhlet apparatus. The percentage of fat in almond seeds was found to be 58.9% and Cucurbitin protein of pumpkin seeds was found to be 51.2%, when defatted with cold acetone at constant stirring. Fat extraction was performed in duplicate and estimated values are given in (Table 2). Defatted sesame meal contains more sugar and is generally utilized as animal feed and often as manure. This meal has a great potential in combating the protein calories malnutrition because of its high quality and quantity of protein. However there is a need to process the meal carefully for human consumption. (Lai et al., 2017).

Estimation of 11s globulin protein of sesame seeds in different NaCl elution

The 11S globulins or legumin-like proteins are members of the cupin superfamily which are characterized by a 6 stranded β-barrel conformation (Rosental et al., 2014). They are typically hexameric ~360 kDa proteins in which each monomeric subunit is comprised of an acidic 40~42 kDa polypeptide that is disulfide-linked to a 20 kDa basic polypeptide. In the plant, seed storage proteins serve as amino acid reserves for the developing seedling and act as a

carbon and nitrogen source during germination (Rosental et al., 2014). Seed storage proteins are abundant in the seed and it is estimated that they can account for ~40% of the total protein (López-Pedrouso et al., 2014). The 11S globulin, amandin and cucurbitin protein was purified by ion exchange column chromatography. DEAE-sepharose anion exchange column was used for purification of 11S globulin protein. The respective protein was eluted by using different concentrations of NaCl. The concentration of protein in different NaCl elutes was estimated from the standard BSA linear equation (Y = 0.091X) and was found to be higher in 400 mM NaCl elute. Estimated values are given in (Table 2).

SDS-PAGE analysis of 11S globulin protein of sesame seeds, amandin protein of almond and cucurbitin protein of pumpkin seeds

The Protein samples were loaded into gel wells and electrophoresis was carried out at a constant current of 100 mA on 15% acrylamide gel. SDS-PAGE analysis of protein samples treated with reducing agents (β-mercaptoethanol) revealed that the isolated 11S globulin protein was composed of acidic polypeptide (30 − 33 kDa) and a single basic polypeptide (20 − 24 kDa) (Fig. 1A), isolated amandin protein was composed of acidic polypeptide (40 − 42 kDa) and a single basic polypeptide (15 − 20 kDa) (Fig. 1B) and isolated cucurbitin protein was composed of acidic polypeptide (35 − 42 kDa) and a basic polypeptide (20 − 25 kDa) (Fig. 1C). This indicated the presence of disulphide bond between the acidic and basic polypeptide. 11S globulin protein sample of sesame seeds was found to be eluted in 400 mMNacl elute. An image of the sesame11S globulin run under reducing conditions is shown in Fig. 1. Cucurbitin, the major storage protein in pumpkin seed belongs to 11S globulin family, were described by Osborne (Kesari et al., 2017). It is a hexamer with a molecular size of about 350 kDa (Rezig et al., 2013). Hexamer protein contains six monomer subunits. Each monomeric subunit is comprised of an acidic polypeptide (33 kDa) and a basic polypeptide (22 kDa). Pumpkin seeds are consumed directly for human consumption as a snack food in many cultures throughout the world (Wang, 2017). The kernels of pumpkin seeds have been utilized as additives to some food dishes (Raihana et al., 2015). Seeds belonging to cucurbitaceae family are known to be as rich in oil as soybean, cottonseed, or corn (Khairi et al., 2014).

Defatted pumpkin seed cake, which is a by-product of oil extraction, is usually used only as animal feed. However, pumpkin oil cake is rich in protein which could be used for human nutrition. The protein exploitation from pumpkin oil cake has been described as a way to increase the value of this agricultural by-product (Veronezi and Jorge, 2015). Oilseed proteins are used in food formulations as nutrition supplements, as well as functional agents (Ejike et al., 2017). Protein bodies are widely distributed in the seeds of higher plants. They are membrane-bound organelles in which various seed proteins are stored. Three major proteins, 11S globulins, 7S globulins and 2Salbumin, are present in the protein bodies of pumpkin seeds. 11S globulin (cucurbitin) forms crystalloids in the protein bodies, while 7S globulin and 2S albumin are located in the matrix of the protein bodies (Hegedus et al., 2015). Amandin is an 11S globulin legumin-like protein that is hexameric in form and composed of individual polypeptides (Bojórquez-Velázquez et al., 2016). Each monomeric subunit, like those of other 11S globulins, is

comprised of a large 40 − 42 kDa acidic chain and a small 20 − 22 kDa basic chain that are linked by a disulfide bond (Natarajan et al., 2013).Two isoforms have been identified in almond, prunin 1 and prunin 2, which comprise native prunin (de la Cruz et al., 2017). Analyses of almond protein extract has been extensively carried out and it was found that prunin is a major component of the nut, accounting for up to 65% of the protein in the soluble extract (da Costa et al., 2013). Analysis revealed that the overall structure of amandin is typical of the 11S globulin class of seed storage proteins, as it is composed of two prunin trimers that associate to form the native hexameric structure (Dhillon et al., 2016).

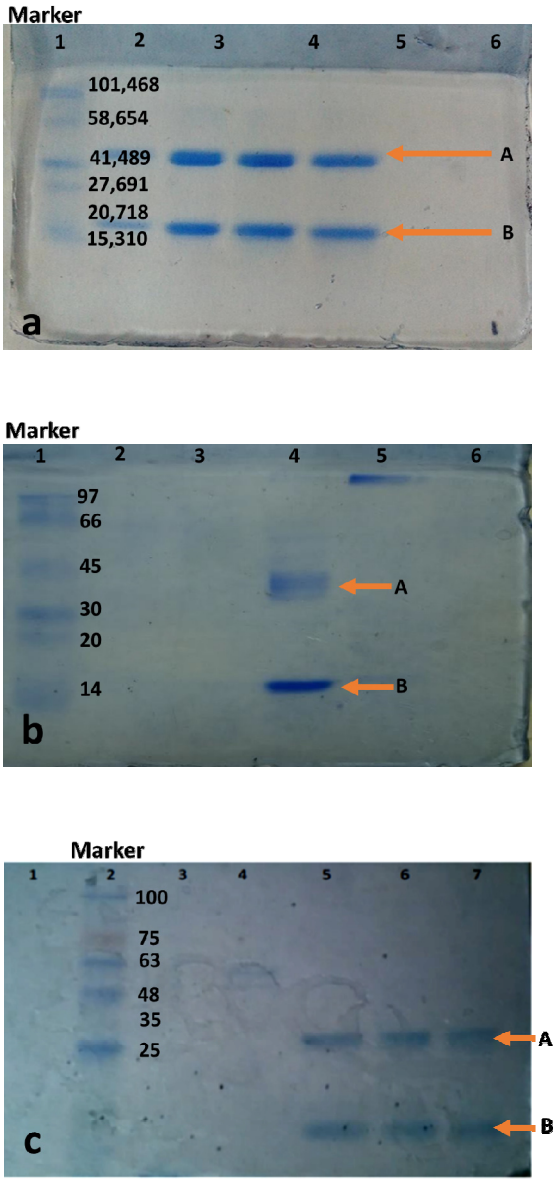

Fig. 1. SDS-PAGE of sesame 11S globulin, almond amandin protein, cucurbitin protein of pumpkin seed samples under reducing conditions (Lane 1: Marker, Lane 2: 100 mM Nacl elute, Lane 3: 200 mM Nacl elute, Lane 4: 400 mM Nacl elute, Lane 5: 600 mM Nacl elute, Lane 6: 1 M Nacl elute; A: Acidic polypeptide, B: Basic polypeptide).

MALDI-TOF

It is a very sensitive technique for determining the mass of proteins or peptides. Protein masses are identity of proteins and thus MALDI allows protein identification. Protein band from SDS-PAGE gel was selected for MALDI TOF analysis. The proteins were subjected to in-gel digestion of trypsin and was analysed by MALDI-TOF to get m/z values and spectrum of cleaved peptides. Protein can be confirmed based on the m/z values of cleaved peptides and peak values obtained from the MALDI-TOF. The m/z values and peak obtained from MALDI-TOF was analyzed using Bioinformatics' tool PROFOUND. The m/z and the peak values obtained have given the best hit for 11S globulin of sesame seed. This confirms that the isolated protein from sesame seed was 11S globulin (Fig. 1A and Fig. 2).

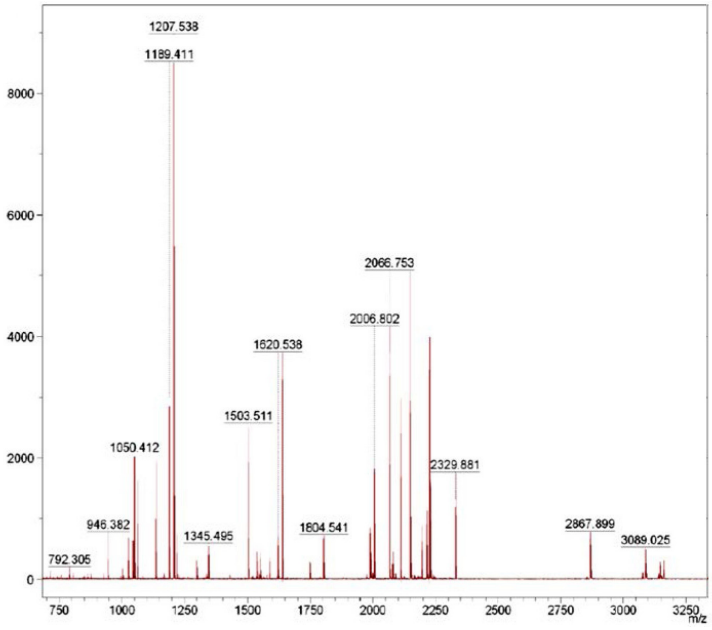

Fig. 2. Matrix Assisted Laser Desorption/Ionization (MALDI) - spectrum of 11S globulin of sesame seed protein.

In-vitro alpha amylase inhibiting assay

Alpha amylase inhibition assay OF 11S globulin protein of sesame seed

Amylase inhibitors are also known as starch blockers because they contain substances that prevent dietary starch from being absorbed by the body. Starch are complex carbohydrates that cannot be absorbed unless they are first broken down by the digestive enzyme amylase and other, secondary enzymes (Kumar et al., 2015).The alpha amylase inhibiting activity of 11S globulin protein of sesame seed was investigated using alpha amylase inhibition assay. The percentage of inhibition was determined for different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/mL) of 11S globulin protein samples. Protein sample of 1 mg/mL has shown maximum percentage of inhibition. The percentage of inhibition for 1 mg/ml almond seed sample was found to be 82.6%, the percentage of inhibition for 1 mg/mL protein sample was found to be 78% (Table 2). Alpha amylase inhibiting activity was

increased as the concentration of protein increased. This shows that the isolated proteins have good inhibiting activity. Estimated percentage of alpha amylase inhibition by different concentrations of 11S globulin protein of sesame seed was given in Table 2. Pancreatic alpha amylase is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose, and a number of α-(1-6) and α-(1-4) oligoglucans. These are then acted on by α-glucosidases and further degraded to glucose which on absorption enters the blood-stream. Degradation of this dietary starch proceeds rapidly and leads to elevated PPHG (post-prandial hyperglycemia). It has been shown that activity of HPA (human pancreatic α-amylase) in the small intestine correlated to an increase in post-prandial glucose levels, the control of which is therefore an important aspect in treatment of type 2 diabetes. Hence, retardation of starch digestion by inhibition of enzymes such as α-amylase plays a key role in the control of diabetes. Inhibitors of pancreatic α-amylase delay carbohydrate digestion causing a reduction in the rate of glucose absorption and lowering the post-prandial serum glucose levels. An effective strategy for type 2 diabetes management is the strong inhibition of inhibition of pancreatic α-amylase (Lin et al., 2016).

Antihypertensive assay- chorioallantoin Membrane assay

Vasodilating effect of protein sample was studied using chrioallantoin membrane (CAM) assay .The CAM is an extraembryonic membrane formed on day 4 of incubation by fusion of the chorion and the allantois. Blood vessels associated with overlying chrionic epithelial cells grow very rapidly. Effect of SpNO (spermnine Nonoate), Adrenaline and protein sample on blood vessels were evaluated. SpNO causes vasodilation, is used as positive control, Adrenaline causes vasoconstriction, is used as negative control (Fig. 3 and Fig. 4A − F).It is postulated that the mechanism of ACE inhibition involves inhibitor interaction with an anionic binding site that is distinct from the catalytic sites (Haque and Chand, 2008).These ACE inhibiting peptides had limited pharmacological application because of their lack of oral activity. Many ACE inhibiting substances have been proposed by pharmaceutical industry as drugs in treatment of hypertension such as captopril. Several side effects such as increased potassium level, reduced renal function, cough, skin, rashes, fetal abnormalities, etc., have been associated with synthetic organic drugs (Kishore, 2013). Therefore various food proteins derived biofunctional peptides have been isolated

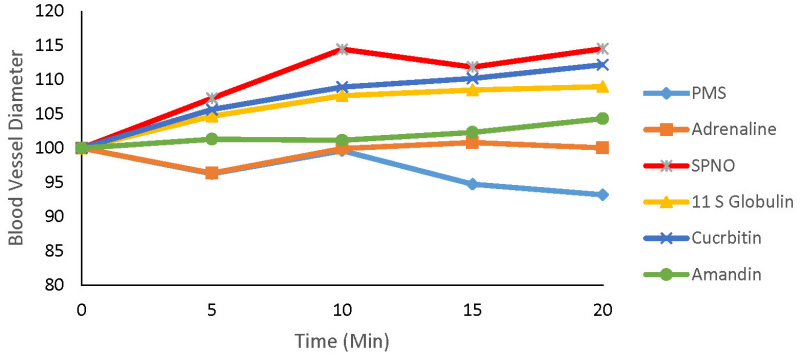

Fig. 3. Chorioallantoin membrane assay - diameter of blood vessel at different time intervals of incubation.

and evaluated for their antihypertensive activity to avoid undesirable side effects of synthetic antihypertensive drugs and to avoid increasing cost of drug therapy. These peptides are mainly derived from: cheese whey, casein, zein, soyabean, fish muscle and gelatine (Brandelli et al., 2015).ACE also removes the C-terminal dipeptide from bradykinin (potent vasodilatator) resulting in the formation of inactive peptide fragments (Haque and Chand, 2008).

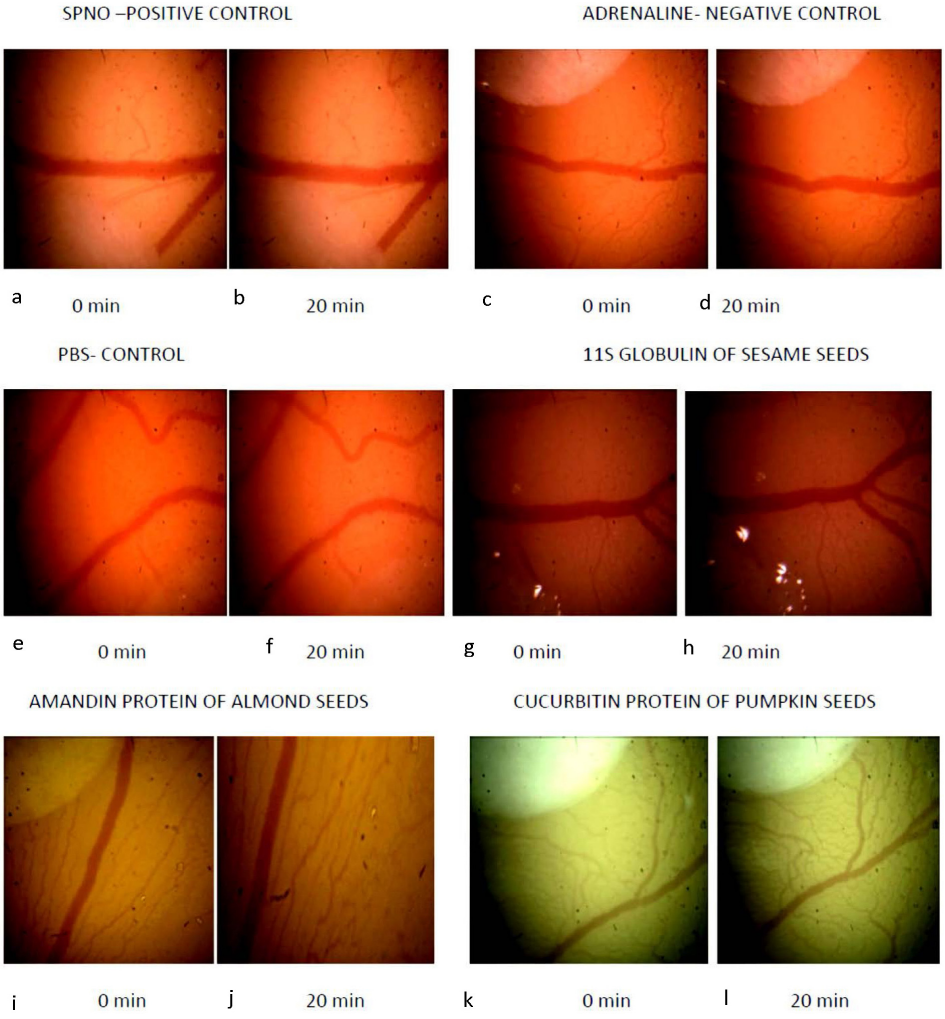

Fig. 4. Chorioallantoin membrane assay - vasodilating effect in chick eggs of Spermine Nonoate (SpNO), adrenaline, PBS, 11S globulin, amandin and cucurbitin proteins. a: Vasodilating effect of SpNO initial time period (positive control). b: Vasodilating effect of SpNO 20 min after incubation at 37 ± 5°C (positive control) blood vessels got dilated. c: Vasodilating effect of adrenaline initial time period (negative control). d: Vasodilating effect of adrenaline 20 min after incubation at 37 ± 5°C (negative control) blood vessels not dilated. e: Vasodilating effect of PBS initial time period. f: Vasodilating effect of PBS 20 min after incubation at 37 ± 5°C. g: Vasodilating effect of 11S globulin (sesame) initial time period. h: Vasodilating effect of 11S globulin (sesame) 20 min after incubation at 37 ± 5°C (blood vessels got dilated). i: Vasodilating effect of prunin (almond seed) protein initial time period. j: Vasodilating effect of prunin (almond seed) protein 20 min after incubation at 37 ± 5°C (blood vessels got dilated). k: Vasodilating effect of cucurbitin (pumpkin) protein initial time period. l: Vasodilating effect of cucurbitin (pumpkin) protein 20 min after incubation at 37 ± 5°C (blood vessels got dilated).

Vasodilatating effect of 11s globulin, cucurbitin and amandin protein

Vasodilatating effect of 11S globulin, cucurbitin and amandin protein (Fig. 4G − L) were evaluated by measuring the diameter of blood vessel at different time intervals. Estimated blood vessel diameter in percentage is given in Fig. 4. Diameter of blood vessel in percentage at different time intervals. The antihypertensive activity is related to the vasodilating effect of protein sample. SpNO (spermine nonoate) is used as NO donor that releases NO extracellularly. SpNO (spermine nonoate) induces vasodilatation through sGC independent mechanism (Fig. 4A − B). Nitric oxide (NO) is a powerful endogenous vasodilator that inhibits platelet aggregation, smooth muscle proliferation, and platelet and monocyte adhesion to the vascular wall (Förstermann et al., 2017). Delivery of exogenous NO is an attractive therapeutic option in the management of these conditions. Organic nitrates and sodium nitroprusside (SNP) are currently used for this purpose, but both have limitations. The diazeniumdiolates (NONOates) constitute a novel class of NO donor drug that decomposes spontaneously to generate up to 2 molecules of NO at physiological pH and temperature (Lee et al., 2014).

SpNO showed increase in blood vessel diameter and adrenaline showed decrease in blood vessel diameter (Fig. 4C − D) than PBS (Phosphate buffer saline pH 7.4) control (Fig. 4E − F). SPER/NO-mediated vasodilatation and inhibition of platelet aggregation both correlate closely with the amount of NO generated. It is generally accepted that the vasodilator effects of NO are mediated via activation of smooth muscle cell soluble guanylate cyclase (sGC) to generate cyclic guanosine-3,5- monophosphate (cGMP).7 However, vasodilatation in response to high concentrations of NO is not exclusively cGMP mediated (Chin et al., 1998).

Blood vessel diameter increased with 11S globulin, cucurbitin and amandin protein sample than PBS control. Vasodilating effect of 11S globulin, cucurbitin and amandin protein sample were higher than PBS but lesser than SpNO (Fig. 4A − L). Vasodilating effect was higher for cucurbitin protein than 11S globulin and amandin protein. This confirms that the 11S globulin, cucurbitin and amandin protein sample have antihypertensive activity. Potential nitric oxide-mediated sGC-independent mechanisms, at high concentrations of SpNo, sufficient No is released extracellularly to react with molecular oxygen. This reaction can form nitrosating species that interact with sulfhydryl-containing molecules. Nitrosation of thiol-containing residues in enzymes has been shown to regulate their function. Therefore, S-nitrosation of thiol-containing enzymes and ion channels, including voltage-sensitive calcium channels and calcium-dependent potassium channels represents a sGC-independent mechanism (Fig. 4G − L). The sGC-independent vasodilatation with SPER/NO may be mirrored by endothelium-derived NO in healthy blood vessels or in NO therapy. The sGC-inhibitor [1H-[1, 2, 4] oxadiazole [4, 3-a] quinoxaline-1-one (ODQ), (Cossenza et al., 2014) a sGCindependent component of vasodilatation has been identified in response to several NO donors. NO have also been shown to have a vasodilator effect in the presence of ODQ, despite complete abolition of cGMP generation (Zhang, 2014).

In summary, SPER/NO releases NO extracellularly and induces vasodilatation that is entirely dependent on NO, but not exclusively mediated by sGC. sGC independent vasodilatation is mediated through the reaction of NO with molecular oxygen, forming higher nitrogen oxides that can regulate the function of thiol-containing proteins

through S-nitrosation. (Z)-1-{N-[3-Aminopropyl]-N-[4-(3 aminopropylammonio) butyl] - amino}-diazen-1-ium-1, 2-diolate (SPER/NO) contains spermine as the nucleophile and decomposes at a relatively slow rate (half life = 39 minutes; 37°C; phosphate buffer, pH 7.4) (Thomas et al., 1991).

Some NO donors enter smooth muscle cells, decompose to NO, and stimulate sGC (ie, GTN and SNP). NO may be generated in the extracellular space (ie, SPER/NO, GSNO, and D-SNVP), a portion of which will pass directly into smooth muscle cells. Instead, extracellular NO may react with superoxide to form peroxynitrite, which does not possess vasodilator activity in this tissue. NO may also react with molecular oxygen to form higher oxides of nitrogen, which are capable of S-nitrosating thiol containing proteins. This process may be linked to sGC independent vasodilatation.

Conclusion

The current study includes the identification of bioactive peptides from sesame, almond and pumpkin seed protein. 11S globulin from sesame seeds, amandin from almond seeds and cucurbitin protein from pumpkin seeds were extracted by ammonium sulphate precipitation and purified by anion exchange chromatography. 11S globulin, amandin and cucurbitin protein are the major seed storage proteins. The 11S globulin, amandin and cucurbitin proteins sub unit pattern were analysed by SDS-PAGE under reducing conditions. Under reducing conditions sample solubilising buffer was used containing 5% β-mercaptoethanol. It was found that the 11S globulin, amandin, cucurbitin proteins dissociated into acidic and basic polypeptides. This indicated the presence of disulphide bond between the acidic and basic polypeptide. Homology searches revealed the presence of antihypertensive and alpha amylase inhibiting peptides in 11S globulin, amandin and cucurbitin proteins. 11S globulin protein of sesame seed was confirmed by MALDI-TOF. Alpha amylase inhibiting activities of proteins were evaluated and it was found that the cucurbitin protein has higher inhibiting activity of about 86% than amandin and 11S globulin protein. Antihypertensive activity of protein samples was measured by vasodilatation of blood vessels in chick embryo. Antihypertensive activity was found to be higher for cucurbitin than 11S globulin and amandin protein.

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