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Research Article

PURIFICATION AND CHARACTERIZATION OF 11S GLOBULIN FROM KUTAJ (*HOLARRHENA ANTIDYSENTERICA*)

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Abstract: Globulins are major seed storage proteins which determine the nutritional quality of the seeds. They form the main source of essential amino acids in the human dietary. The seeds of the Kutaj plant are used in the treatment of various diseases and disorders. Transgenic approaches can be applied to rectify or enhance the content of essential amino acids in seed proteins. It necessitates the study and characterization of globulins from various plant families and sources. Herein, we report the *Holarrhena antidysenterica* globulin (11S globulin) purified from seeds of Indian medicinal plant, Kutaj. Pure HAG protein was obtained by using DEAE anion-exchange followed by size-exclusion chromatography. The protein exists as a hexamer of ~350 kDa. The SDS-PAGE gel in reducing condition showed bands at ~36 kDa (α subunit) and ~22 kDa (β subunit); and non-reducing condition ~58 kDa which suggest the presence of disulphide bond between the α and β subunit. The N-terminal amino acid sequence of the larger subunit is LRQPQLNEAQ and shows high sequence homology with already known 11S seed storage globulin. Molisch's test indicated that it is a glycoprotein. Glycosylation phenomenon in 11S globulin helps in protein transport from ER to the vacuolar bodies.

Keywords: *Holarrhena antidysenterica;* seed protein; 11S globulin; lectin like protein; Glycosylation

Note : Coloured Figures and Supplementary Information available on Journal Website in "Archives" Section

Introduction

Holarrhena antidycenterica belongs to the family *Apocynaceae;* called Tellicherry bark in English and Kutaj in Sanskrit, a well known medicinal plant. It is a native tropical plant found in areas of Africa and Asia. Seeds and bark of the plant have been used in the treatment of various diseases and disorders particularly for dysentery, diarrhoea, amoebiasis, piles, intestinal worms, fever, cold, skin diseases and biliousness (Chakraborty *et al.*, 1999; Gautam *et al.*, 2007; Khan *et al.*, 2001). Various alkaloids have been isolated from H. *antidycenterica* and have been shown to possess antidysentric, antibacterial, antidiarrhoeal, immunomodulatory and insecticidal properties (Chakraborty and Brantner, 1999; Kavitha *et al.*, 2009; Kavitha *et al.*, 2004; Thappa *et al.*, 1989). Seeds of medicinal plants are rich in alkaloids, proteins, peptides that possess antimicrobial and insecticidal properties. Various proteins such as protease inhibitors, alpha-amylase inhibitors, proteases, lectins, globulins, chitinase and albumins have been isolated from medically important plants and studied for their potential application in medicinal and agriculture industries (Kesari *et al.*, 2015; Kumar *et al.*, 2012; Patil *et al.*, 2009; Patil *et al.*, 2012; Patil *et al.*, 2009; Patil *et al.*, 2013; Singh *et al.*, 2015; Tomar *et al.*, 2014; Tomar *et al.*, 2009).

Plant seeds contain various kinds of storage proteins including large globular proteins called

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globulins or legumin-like proteins that act as a source of nutrition for the new embryos during seed germination, thus play a pivotal role in the seed germination process. The 11S seed legumin-like proteins are hexameric hetero-oligomers with molecular weight of ~ 300-400 kDa, with each protomer sub-divided into an acidic and basic subunit that are associated by disulfide linkage (Adachi *et al.*, 2003; Jung *et al.*, 1998; Shewry, 1995). These 11S globulins are synthesized as single precursor polypeptide which is proteolytically post-translationally processed by signal peptidase and asparaginyl endopeptidase that results in the formation of the acidic and basic domains of the 11S protomer that interact to form hexamer (Adachi *et al.*, 2003; Jung *et al.*, 1998; Shewry *et al.*, 1995).

Globulins along with albumins are the major seed storage proteins of food crops i.e. cereals and legumes, and are also present in abundance in nuts. These proteins have been isolated and characterized from a variety of plant species including *Pisum sativum* (Rangel *et al.*, 2003), *Vicia faba* (Wright *et al.*, 1974), *Arabidopsis thailiana* (Jaworski *et al.*, 2014), *Glycine max* (Yagasaki *et al.*, 1997), *Avena sativa* (Shotwell *et al.*, 1988), *Actinidia deliciosa* (Rassam *et al.*, 2006), *Triticum aestivum* (Burgess *et al.*, 1986), *Oryza sativa* (Komatsu *et al.*, 1992), *Ginkgo biloba* (Jin *et al.*, 2008), *Coffea arabica* (Coelho *et al.*, 2010), *Arachis hypogaea* (Marsh *et al.*, 2008), *Prunus dulcis* (Jin, 2009) and *Corylus avellana* (Beyer *et al.*, 2002) etc. Being one of the major storage proteins in various foods, globulins play an important role in nutrition of humans and farm animals by providing essential amino acids. However, some of these globulins including 11S globulin, have been identified as the major food allergens. In addition to the role of globulins as seed storage proteins, they have been shown to have additional secondary activities such as protease inhibitory activity (Rassam and Laing, 2006), insecticidal activity (Macedo *et al.*, 2000; Soares, 2007), chitin-binding (Moura *et al.*, 2007) and lectin-like activity (Soares, 2007). Due to these activities, globulins have also been implicated to play a defensive role in plant pathogenesis (Coelho *et al.*, 2010).

Transgenic crop plants are being generated for nutritional improvement or increased productivity and are being potentially employed in the agriculture industry (Jaworski and Aitken, 2014; Shewry *et al.*, 2008). Moreover, PR proteins which are involved in plant defense mechanism help in generating disease and pest resistant crops plants (Murdock *et al.*, 2002). Globulins are rich in essential amino acids and have characteristics of PR proteins. Their investigation help in construction of transgenic plants with dual behaviour (Beyer *et al.*, 2002; Bright *et al.*, 1983; Coelho *et al.*, 2010; Jin, 2009; Shewry *et al.*, 2008; Soares, 2007). Therefore, it becomes necessary to study and characterize globulins from various plant families and sources.

In the present study, we report isolation, purification and characterization of 11S HAG from Kutaj seeds. HAG was fractionated in two steps through anion-exchanger and size-exclusion chromatography columns. Isolated fractions were examined under reducing and non-reducing conditions to determine the molecular weights of their constituent polypeptides. The N-terminal sequence of larger subunit of HAG was determined and sequence homology search was performed to identify purified protein.

Material and Methods

Dry seeds of *Holarrhena antidysenterica* were obtained from local market. The reagents used for the purification and experimental assays were purchased form Sigma-Aldrich corp, St. Louis, MO USA; BioRad Laboratories, Hercules, California, USA; Himedia Laboratories India private limited, Mumbai, India; Merck Limited, Worli, Mumbai, India. HiLoad Superdex 200 16/60 column, HMW Calibration kit from GE Health care Bio science, AB Uppsala, Sweden. Amicon ultra Concentrator, PVDF membrane & millex syringe filter from Millipore corporation, Billerica, MA. 3500 MW cut off dialysis membrane from Pierce, Rockford, USA.

Extraction and Purification of HAG

Seeds (5.0 g) were soaked overnight at room temperature in 20 ml buffer A (50 mM Tris-HCl, pH 7.5). Seed coat was removed manually and seed kernels were obtained. A crude extract was prepared by homogenizing kernels in buffer B (50 mM Tris-HCl, pH 7.5, 10% glycerol, 10 mM $MgCl_2$,

0.1 mM PMSF) using a mortar and pestle. After stirring for 6 hr at 4 °C, the seed extract was centrifuged at 20,000 rpm, 4 °C for 45 min. After centrifugation, the uppermost layer of fat was discarded and the sample was again centrifuged at 20,000 rpm, 4 °C for 45 min. The clear supernatant collected after centrifugation was subjected to chromatography on a 5 ml DEAE anion-exchanger column which had been pre-equilibrated with buffer A. Following elution of unbound proteins in the flow through, the column was washed with 25 ml of buffer A and bound material was subsequently eluted from the column with a step gradient of NaCl from 0.1 M-0.5 M NaCl (0.1, 0.2, 0.3, 0.4 and 0.5 M) in buffer A. The fractions obtained from DEAE chromatography were analyzed on 12% SDS-PAGE and fractions containing HAG were pooled and concentrated to 10 mg/ml using Amicon Ultra 15.

The concentrated protein sample was finally loaded onto pre-equilibrated HiLoad 16/60 Superdex 200 size-exclusion column. Fractions of the major peak containing pure protein were pooled and concentrated using Amicon ultra 15. The purity of the concentrated HAG sample was determined by 12% SDS–PAGE stained with Coomassie Brilliant Blue. Protein concentration was determined by using Bio-Rad protein assay kit and taking BSA as standard. Purified and concentrated protein was dialyzed overnight against the dialysis buffer (50 mM Tris-HCl, pH 7.5) and stored at -20 °C.

Amino Terminal Sequencing

The Edman degradation was performed on an automated protein sequencer (model 494; Applied Biosystems) at the sequencing facility at Columbia University, New York, USA for deducing N-terminal amino acid sequence of larger subunit of HAG. Pure protein was subjected to a 12% SDS-PAGE under reducing condition and was electroblotted onto a polyvinylidene fluoride (PVDF) membrane using 10 mM CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) buffer, pH 11 in 10% methanol (Matsudaira, 1987). The band corresponding to the larger α-subunit was excised from PVDF membrane and the first 10 amino acid residues at the N-terminal of the excised polypeptide band were determined. The obtained amino acid sequence was subjected to *BLAST* search against PDB database (*http://www.ncbi.nlm.nih.gov/BLAST*) for identification of purified protein based on sequence homology.

Glycosylation Assay

The glycosylated nature of the purified protein was first confirmed by Molisch Test (Dreywood, 1946). It is qualitative test wherein the solution of anthrone in concentrated sulfuric acid gives a permanent green coloration in the presence of sugar.

Results and Discussion

Extraction and purification of HAG

HAG was purified successfully to homogeneity in two steps by DEAE anion-exchange and Superdex 200 size-exclusion chromatography. In the first step, the fractions containing protein were eluted in 100 mM to 500 mM NaCl gradient and the presence of the protein in these fractions was analyzed on 12% SDS-PAGE **(Figure 1)**. In size exclusion chromatography step, the protein

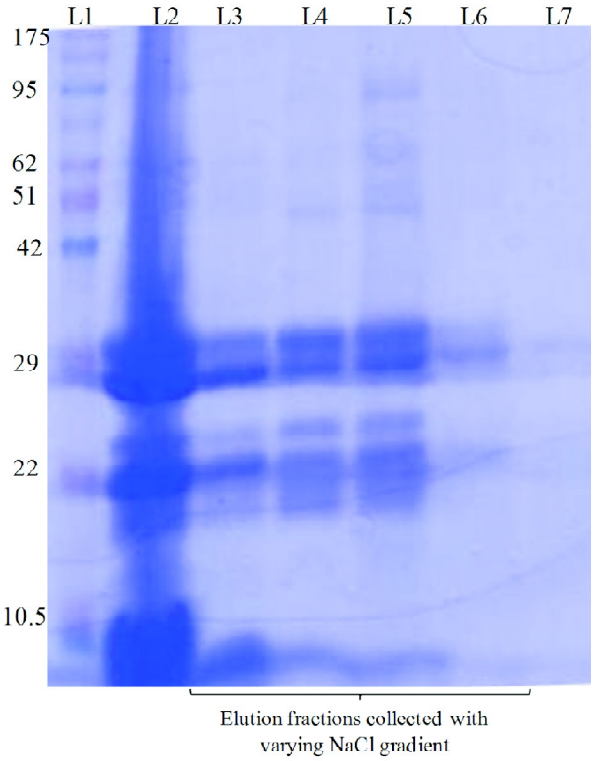

Figure 1: **SDS-PAGE profile of DEAE chromatography step. Lane 1: Molecular weight marker (kDa); Lane 2: Supernatant; Lane 3-7: 100 mM to 500 mM NaCl gradient.**

eluted at a volume ~53 ml as shown in the chromatogram **(Figure 2)**. The purity of protein in the major peak fractions were analyzed on SDS-PAGE. Major peak fractions showed a single band of ~58 kDa in non-reducing condition and in the presence of reducing agent (β-mercaptoethanol), two subunits of ~36 kDa and ~22 kDa were observed **(Figure 3)**.

Literature shows 11S globulins members exist in hexametric state as found in Arachin from *Arachis hypogea* (330-350 kDa) (Johnson *et al.*, 1950), Conglutin α from *Lupines spp.* (330–430 kDa) (Nadal *et al.*, 2011), Cruciferin from *Brassica napus* (~ 300–390 kDa) (Nietzel *et al.*, 2013), Cucurbitin from *Cucurbita spp* (340-380 kDa) (Hara-Nishimura *et al.*, 1985), Glycinin from *Glycine max* (320-375 kDa) (Barton *et al.*, 1982), Helianthinin from *Helianthus annulus* (300-350 kDa) (Schwenke *et al.*, 1979), legumin from *Pisum sativum* (330-450 kDa); and globulins from *Vicia faba* (320-400 kDa) and *Vigna unguiculata* (300-400 kDa) (Ersland *et al.*, 1983). Comparing the molecular weight of HAG oligomer form (~350 kDa) with other known members showed that HAG also exists as a hexamer in natural state.

N-terminal sequencing

The purified HAG after reducing SDS-PAGE was electroblotted onto PVDF and N-terminal sequence of HAG was determined. The obtained sequence of first 10 residues was Leu-Arg-Gln-Pro-Gln-Leu-Asn-Glu-Ala-Gln. This sequence was used for homology search using BLASTP against PDB database. The polypeptide showed high similarity with N-terminal sequences of 11S seed globulin from Pea Prolegumin and Soybean Proglycinin (Adachi *et al.*, 2001; Tandang-Silvas *et al.*, 2010) **(Figure 4)** and confirmed that the isolated protein is an 11S globulin.

Glycosylation assay

Glycosylation not only promotes protein stabilization but also helps in protein trafficking. The initial translation product of globulin is produced in the ER, from where they are then transported to vacuoles for hexamer formation. The vacuole surface contains lectins, which recognize the sugar present on globulin surface; thereby allowing globulins to enter the vacuoles (Duranti *et al.*, 1995).

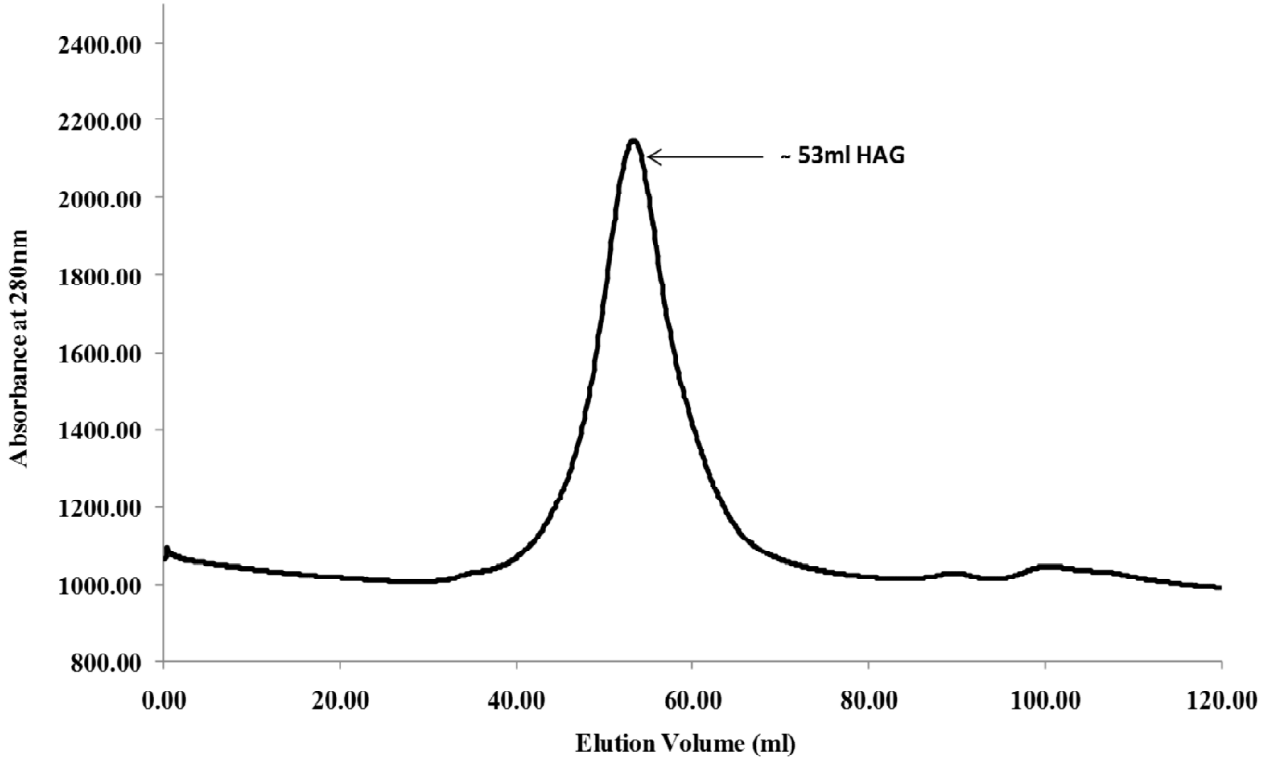

Figure 2: **Chromatogram of HAG after size exclusion chromatography step. The peak fractions corresponding to elution volume ~53 ml indicate the hexameric form of HAG.**

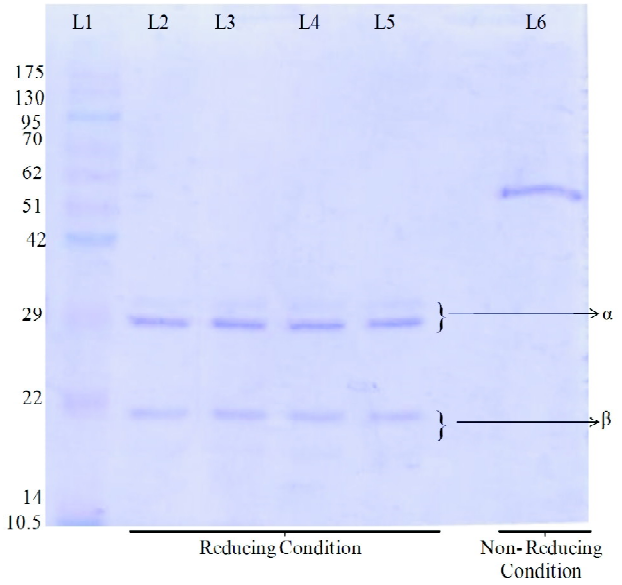

Figure 3: **12% SDS-PAGE profile of HAG fractions after size exclusion chromatography. Lane 1: Molecular weight marker (kDa); Lane 2-5: Two bands of ~36 kDa and ~22 kDa in reducing condition, where disulphide bridges get reduced in presence of reducing agent which determines the hetrodimeric nature of HAG monomer; Lane 6: Pure protein single band of ~58 kDa in non-reducing condition.**

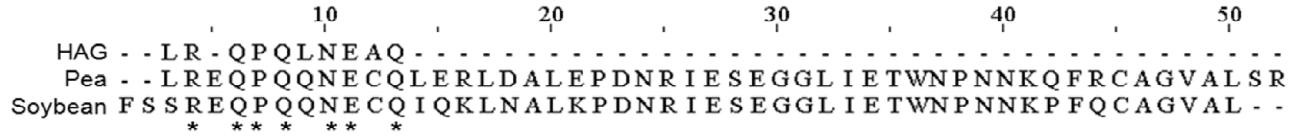

Figure 4: **N-terminal amino acid sequence alignment of 11S globulin from *H. antidysenterica* with other known 11S globulin members like prolegumin from *Pisum sativum* (PDB ID: 3KSC) and proglycinin A1AB1B homotrimer from *Glycine max* (PDB ID: 1FXZ).**

Generally, seed storage proteins are glycosylated and HAG protein is one of the major seed protein of *H. antidysenterica*. Therefore, the presence of carbohydrate moiety in purified protein was tested using Molisch test. The test indicated that it is a glycoprotein. Glycosylation of 11S globulin is rare, but it has been reported for 11S globulins from coconut (Garcia *et al.*, 2005) and lupin seeds (Duranti *et al.*, 1988; Duranti *et al.*, 1995).

Conclusions

11S globulins dominate the protein content of seeds. They act as rich source of essential amino acids in the human dietary. Protein engineering studies to alter the physicochemical properties of this protein can help in enhancing the nutritional quality of seeds. However the limiting factor of such studies is that the recombinant expression of such protein results in accumulation of the variants with improper folding and/or reduced stability (Galili *et al.*, 2013; Müntz, 1998). Thus understanding the factors involved in its stability and transport in the cell will help in increasing its production. Our study show that HAG purified from the seeds of *Holarrhena antidycentrerica* is a member of 11S globulin family. The molecular weight of HAG hexamer is ~350 kDa which is comparable to weight of other known 11S members. In the vacuoles, the cleavage by protease and dimerization of trimers leads to formation of a hexamer. The compact hexameric scaffold of globulin promotes its long term storage. Molisch test indicates that the protein is glycosylated. Glycosylation plays an important role by influencing the structure and function of proteins.

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Abbreviations

BSA, bovine serum albumin; DEAE, Diethyl amino ethyl; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PDB, Protein data bank. HMW, high molecular weight.

Conflict of Interest

The authors do not have any conflict of interest of this manuscript.

References

Adachi, M., Takenaka, Y., Gidamis, A.B., Mikami, B., and Utsumi, S. (2001). Crystal structure of soybean proglycinin A1aB1b homotrimer. J Mol Biol, *305,* 291-305.

Adachi, M., Kanamori, J., Masuda, T., Yagasaki, K., Kitamura, K., Mikami, B., and Utsumi, S. (2003). Crystal structure of soybean 11S globulin: glycinin A3B4 homohexamer. Proc. Natl. Acad. Sci, *100,* 7395-7400.

Barton, K.A., Thompson, J.F., Madison, J.T., Rosenthal, R., Jarvis, N.P., and Beachy, R.N. (1982). The biosynthesis and processing of high molecular weight precursors of soybean glycinin subunits. J. Biol. Chem, *257*, 6089-6095.

Beyer, K., Grishina, G., Bardina, L., Grishin, A., and Sampson, H.A. (2002). Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. Clin Immunol, *110*, 517-523.

Bright, S.W.J., Shewry, P.R., and Kasarda, D.D. (1983). Improvement of protein quality in cereals. Crit Rev Plant Sci, *1*, 49-93.

Burgess, S.R., and Shewry, P.R. (1986). Identification of homologous globulins from embryos of wheat, barley, rye and oats. J. Exp. Bot, *37*, 1863-1871.

Chakraborty, A., and A.H. Brantner, (1999). Antibacterial steroid alkaloids from the stem bark of *Holarrhena pubescens*. J Ethnopharmacol, *68*, 339-344.

Coelho, M.B., Macedo, M.L.R., Marangoni, S., Silva, D.S., Cesarino, I., and Mazzafera, P. (2010). Purification of Legumin-Like Proteins from *Coffea arabica* and *Coffea racemosa* Seeds and Their Insecticidal Properties toward Cowpea Weevil (*Callosobruchus maculatus*) (Coleoptera: Bruchidae). J. Agric. Food Chem, *58*, 3050-3055.

Dreywood, R. (1946). Qualitative test for carbohydrate material. ýInd. Eng. Chem. Res, *18*, 499-499.

Duranti, M., Guerrieri, N., Takahashi, T., and Cerletti, P. (1988). The legumin-like storage protein of *Lupinus albus* seeds. Phytochem, *27*, 15-23.

Duranti, M., Horstmann, C., Gilroy, J., and Croy, R.R.D. (1995). The molecular basis for N-glycosylation in the 11S globulin (legumin) of lupin seed. J Protein Chem, *14*, 107-110.

Ersland, D.R., J.W.S. Brown, R. Casey, and T.C. Hall, (1983). The Storage Proteins of *Phaseolus vulgaris* L., *Vicia faba* L. and *Pisum sativum* L. In Advances in Agricultural Biotechnology, (eds. W. Gottschalk and H. P. Müller,) Springer, Netherlands, pp. 355-375

Galili, G., and Amir, R. (2013). Fortifying plants with the essential amino acids lysine and methionine to improve nutritional quality. Plant Biotech J, *11*, 211-222.

Garcia, R.N., Arocena, R.V., Laurena, A.C., and Tecson-Mendoza, E.M. (2005). 11S and 7S globulins of coconut (*Cocos nucifera* L.): purification and characterization. J. Agric. Food Chem, *53,* 1734-1739.

Gautam, R., Saklani, A., and Jachak, S.M. (2007). Indian medicinal plants as a source of antimycobacterial agents. J Ethnopharmacol, *110*, 200-234.

Hara-Nishimura, I., Nishimura, M., and Akazawa, T. (1985). Biosynthesis and intracellular transport of 11S globulin in developing pumpkin cotyledons. Plant Physiol, *77*, 747-752.

Jaworski, A.F., and Aitken, S.M. (2014). Expression and characterization of the Arabidopsis thaliana 11S globulin family. Biochim. Biophys. Acta, *1844*, 730-735.

Jin, T., Chen, Y.W. Howard, A., and Zhang, Y.Z. (2008). Purification, crystallization and initial crystallographic characterization of the *Ginkgo biloba* 11S seed globulin ginnacin. Acta Crystallogr Sect F Struct Biol Cryst Commun, *64*, 641-644.

Jin, T., Albillos, S.M., Guo, F., Howard, A., Fu, T.J., Kothary, M.H., Zhang, Y.Z. (2009). Crystal structure of prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin. J. Agric. Food Chem, *57*, 8643–8651.

Johnson, P., and Shooter, E.M. (1950). The globulins of the ground nut (*Arachis Hypogaea*): I. Investigation of arachin as a dissociation system. Biochim. Biophys. Acta, *5,* 361-375.

Jung, R., Scott, M.P., Nam, Y.W., Beaman, T.W., Bassüner, R., Saalbach, I., Müntz, K., and Nielsen, N.C. (1998). The role of proteolysis in the processing and assembly of 11S seed globulins. Plant Cell, 10, 343-357.

Kavitha, D., Shilpa, P.N., and Devaraj, S.N. (2004). Antibacterial and antidiarrhoeal effects of alkaloids of Holarrhena antidysenterica WALL. Indian J Exp Biol, *42*, 589-594.

Kavitha, D., and Niranjali, S. (2009). Inhibition of enteropathogenic Escherichia coli adhesion on host epithelial cells by *Holarrhena antidysenterica* (L.) WALL. Phytother. Res, *23,* 1229-1236.

Kesari, P., Patil, D.N., Kumar, P., Tomar, S., and Sharma, A.K. (2015). Structural and functional evolution of chitinase-like proteins from plants. Proteomics *15*, 1693-1705.

Khan, S., and Balick, M.J. (2001). Therapeutic plants of Ayurveda: a review of selected clinical and other studies for 166 species. J. Altern. Complement. Med, *7,* 405-515.

Komatsu, S., and Hirano, H. (1992). Rice seed globulin: a protein similar to wheat seed glutenin. Phytochem, *31,* 3455-3459.

Kumar, P., Patil, D.N., Chaudhary, A., Tomar, S., Yernool, D., Singh, N., Dasauni, P., and Kundu, S. (2012). Purification and biophysical characterization of an 11S globulin from Wrightia tinctoria exhibiting hemagglutinating activity. Protein Pept Lett, *20*, 499-509.

Macedo, M.L.R., Coelho, M.B., Freire, M.G.M., Machado, O.L.T., Marangoni, S., and Novello, J.C. (2000). Effect of a toxic protein isolated from *Zea mays* seeds on the development and survival of the cowpea weevil, *Callosobruchus maculatus*. Protein Pept Lett, *7*, 225-232.

Marsh, J., Rigby, N., Wellner, K., Reese, G., Knulst, A., Akkerdaas, J., R. van Ree, Radauer, C., Lovegrove, A., and Sancho, A. (2008). Purification and characterisation of a panel of peanut allergens suitable

for use in allergy diagnosis. Mol Nutr Food Res, *52*, 272-285.

Matsudaira, P., (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem, *262*, 10035-38.

Moura, F.T., Oliveira, A.S., Macedo, L.L.P., Vianna, A.L.B.R., Andrade, L.B.S., Martins-Miranda, A.S., Oliveira, J.T.A., Santos, E.A., and Mauricio, P. (2007). Effects of a chitin-binding vicilin from *Enterolobium contortisiliquum* seeds on bean bruchid pests (*Callosobruchus maculatus* and *Zabrotes subfasciatus*) and phytopathogenic fungi (*Fusarium solani* and *Colletrichum lindemuntianum*). J Agric Food Chem, *55*, 260-266.

Müntz, K., (1998). Deposition of storage proteins, In Protein Trafficking in Plant Cells, (ed. J. Soll), Springer, Netherlands, pp. 77-99.

Murdock, L.L., and Shade, R.E. (2002). Lectins and protease inhibitors as plant defenses against insects. J Agric Food Chem, *50*, 6605-6611.

Nadal, P., Canela, N., Katakis, I., and O'Sullivan, C.K. (2011). Extraction, isolation, and characterization of globulin proteins from *Lupinus albus*. J Agric Food Chem, *59*, 2752-2758.

Nietzel, T., Dudkina, N.V., Haase, C., Denolf, P., Semchonok, D.A., Boekema, E.J., Braun, H.P., and Sunderhaus, S. (2013). The native structure and composition of the cruciferin complex in *Brassica napus*. J Biol Chem, *288*, 2238-2245.

Patil, D.N., Datta, M., Chaudhary, A., Tomar, S., Sharma, A.K., and Kumar, P. (2009). Isolation, purification, crystallization and preliminary crystallographic studies of chitinase from tamarind (Tamarindus indica) seeds. Acta Crystallogr Sect F Struct Biol Cryst Commun, *65*, 343-345.

Patil, D.N., Chaudhry, A., Sharma, A.K., Tomar, S., and Kumar, P. (2009). Purification, crystallization and preliminary crystallographic studies of a Kunitz-type proteinase inhibitor from tamarind (*Tamarindus indica*) seeds. Acta Crystallogr Sect F Struct Biol Cryst Commun, *65*, 736-738.

Patil, D.N., Chaudhry, A., Sharma, A.K., Tomar, S., and Kumar, P. (2012). Structural basis for dual inhibitory role of tamarind Kunitz inhibitor (TKI) against factor Xa and trypsin. FEBS J, *279*, 4547-4564.

Patil, D.N., Datta, M., Dev, A., Dhindwal, S., Singh, N., Dasauni, P., Kundu, S., Sharma, A.K., Tomar, S., and Kumar, P. (2013). Structural investigation of a novel N-acetyl glucosamine binding chi-lectin which reveals evolutionary relationship with class III chitinases. PLoS One, *8*, e63779.

Rangel, A., Domont, G.B., Pedrosa, C., and Ferreira, S.T. (2003). Functional properties of purified vicilins from cowpea (*Vigna unguiculata*) and pea (*Pisum sativum*) and cowpea protein isolate. J Agric Food Chem, *51*, 5792-5797.

Rassam, M., and Laing, W.A. (2006). The interaction of the 11S globulin- like protein of kiwifruit seeds with pepsin. Plant Sci, 171, 663–669.

Schwenke, K.D., Paehtz, W., Linow, K.J., Raab, B., and Schultz, M. (1979). On seed proteins Part 11. Purification, Chemical Composition, and Some Physico-chemical Properties of the 11 S Globulin (Helianthinin) in Sunflower Seed. Mol Nut Food Res, *23*, 241-254.

Shewry, P.R., (1995). Plant storage proteins. Biol Rev, *70*, 375-426.

Shewry, P.R., J.A. Napier, and A.S. Tatham, (1995). Seed storage proteins: structures and biosynthesis. Plant Cell, *7*, 945-956.

Shewry, P., H. Jones, and N. Halford, (2008). Plant biotechnology: transgenic crops. Adv Biochem Eng Biotechnol, *111*, 149-186.

Shotwell, M.A., Afonso, C., Davies, E., Chesnut, R.S., and Larkins, B.A. (1988). Molecular characterization of oat seed globulins. Plant Physiol, *87*, 698-704.

Singh, A., Selvakumar, P., Saraswat, A., Tomar, P.P., Mishra, M., Singh, P.K., and Sharma, A.K. (2015). Characterization and cloning of an 11S globulin with hemagglutination activity from *Murraya paniculata*. Protein Pept Lett, *22*, 750-761.

Soares, E.L., Freitas, C.D.T., Oliveira, J.S., Sousa, P.A.S., Sales, M.P., Barreto-Filho, J.D.M., Bandeira, G.P., Ramos, M.V. (2007). Characterization and insecticidal properties of globulins and albumins from *Luetzelburgia auriculata* (Allemao) Ducke seeds towards *Callosobruchus maculatus* (F) (Coleoptera: Bruchidae). J Stored Prod Res, *43*, 459-467.

Tandang-Silvas, M.R., Fukuda, T., Fukuda, C., Prak, K., Cabanos, C., Kimura, A., Itoh, T., Mikami, B., Utsumi, S., and Maruyama, N. (2010). Conservation and divergence on plant seed 11S globulins based on crystal structures. Biochim Biophys Acta – Proteins and Proteomics, *1804*, 1432-1442.

Thappa, R.K., Tikku, K., Saxena, B.P., Vaid, R.M., and Bhutani, K.K. (1989). Conessine as a larval growth inhibitor, sterilant, and antifeedant from *Holarrhena antidysenterica* Wall. Int J Trop Insect Sci, *10*, 149-155.

Tomar, S., Patil, D.N., Datta, M., Tapas, S., Preeti, Chaudhary, A., Sharma, A.K., and P. Kumar, P. (2009). Crystallization and preliminary X-ray diffraction analysis of the complex of Kunitz-type tamarind trypsin inhibitor and porcine pancreatic trypsin. Acta Crystallogr Sect F Struct Biol Cryst Commun, *65*, 1179-1181.

Tomar, P.P., Chaudhary, N.S., Mishra, P., Gahloth, D., Patel, G.K., Selvakumar, P., Kumar, P., and Sharma, A.K. (2014). Purification, Characterisation and Cloning of a 2S Albumin with DNase, RNase and Antifungal Activities from *Putranjiva roxburghii*. Appl Biochem Biotechnol, *174*, 471-482.

Wright, D.J., and Boulter, D. (1974). Purification and subunit structure of legumin of *Vicia faba* L.(broad bean). Biochem. J, *141*, 413-418.

Yagasaki, K., Takagi, T., Sakai, M., and Kitamura, M. (1997). Biochemical characterization of soybean protein consisting of different subunits of glycinin. J. Agric. Food Chem, *45*, 656-660.