

Characterization and Structural Identification of an 11S Globulin of *Juglans mandshurica* Maxim. Kernel from the Changbai Mountains in China

Li Fang^{1,2}, Xiao-Fei Wang^{1,2}, Chun-Lei Liu^{1,2}, Peng Wang^{1,2}, Wei Liu^{1,2}, Wei-Hong Min^{1,2*}

¹College of Food Science and Engineering, Jilin Agricultural University, Changchun 130118, China.

²National Engineering Laboratory on Wheat and Corn Further Processing, Jilin Agricultural University, Changchun 130118, China.

KEYWORD: *Juglans mandshurica* Maxim.; Walnut; 11S globulin; purification; structural characterization

ABSTRACT: The major seed storage protein 11S globulin from *Juglans mandshurica* Maxim. (JSG) was isolated and purified to identify its structure and analyze its physicochemical properties. JSG was found to be composed of two distinct major polypeptides with molecular masses of ~23 and ~20 kDa, and whose amino acid sequences are highly similar to those of the 11S globulin from *J. regia* in the NCBI database. JSG contains essential amino acids that correspond to the adult standard recommended by FAO/WHO. Differential scanning calorimetry revealed a single endothermic peak with a denaturation temperature of 96.83 °C and an enthalpy value of 12.07 J/g. The secondary structure of the JSG consists of β -sheets (32.83%) and α -helices (11.67%). A red shift was also observed in the fluorescence spectra with guanidine hydrochloride (GdnHCl) or urea as denaturant; GdnHCl was proven to be a stronger denaturant than urea.

INTRODUCTION

Juglans mandshurica Maxim. (JMM) of the family Juglandaceae is used as a source of food and medicine for immunoregulation and brain boosting (Bao 2011). JMM is native to the Lesser Khingan Mountains, Changbai Mountains, and the Liaoning Eastern Mountains in China and is widely distributed in Northeast China. The history of JMM cultivation can be traced back to the 1st century BC (Wei 2009). JMM kernel is rich in unsaturated fatty acids, proteins, carbohydrates, vitamins, and minerals, with small amounts of phenolic acids and flavonoids, and has thus attracted considerable attention because of its high nutritional value (Yu 2012).

Few studies have investigated the proteins from JMM. Bao (2011) reported the extraction process of the water-soluble protein of JMM kernels. Yu (2012) extracted proteins and prepared an antihypertensive peptide from JMM kernels. Sun (2014) isolated albumin, globulin, and glutelin from JMM and characterized their functional properties, such as foaming capacity, emulsion ability, and emulsion stability.

Most nuts contain 11S globulins as major storage proteins (Mandal et al. 2000), which have been isolated and characterized from various plants, including kiwifruit (Rassam and Laing 2006), ginkgo (Jin et al. 2008), coffee (Coelho et al. 2010), peanut (Marsh et al. 2008), and Brazil nut (Sharma et al. 2010). Previous studies found that 11S globulins serve as the nutrient source for emerging embryos in seed germination and provide essential amino acids (EAAs) to humans and farm animals (Kumar et al. 2013; Shewry and Halford 2002). Furthermore, 11S globulins can be used as additives in various food products because of their gelling and caking functionalities (Martins and Netto 2006; Mujoo et al. 2003; Renkema et al. 2001).

11S globulin is the principal component of the seed storage proteins of JMM. The present study aimed to isolate, purify, and characterize the biochemical and structural properties of 11S globulin.

MATERIALS AND METHODS

Materials

JMM kernels were obtained from Dunhua Guang Sheng Gease Biotechnology Co., Ltd. Chromatography columns, DEAE-Sepharose Fast Flow, HiPrep16/60 Sephacryl S-200 HR, and a 0.22 µm membrane were purchased from GE Healthcare, AB Uppsala, Sweden. Millex syringe filters were procured from Millipore Corporation, Billerica, MA. Urea, guanidine hydrochloride (GdnHCl), and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. All other chemicals used were of analytical or chromatographic grade.

Preparation of defatted JMM flour

Shelled JMM kernels were ground and defatted by a Soxhlet extractor with diethyl ether to degrease rosin. The defatted flour was dried overnight in a fume hood, passed through a 40 mesh sieve, and stored in a polyethylene bag at -20 °C until protein extraction.

Extraction and purification of JSG

Approximately 200 g of defatted JMM flour was mixed with distilled water (1:10 w/v) and stirred for 30 min at 4 °C. The mixture was centrifuged at 5,000 g for 10 min at 4 °C, and the supernatant was discarded. The precipitate was collected and soaked in 10 volumes of 50 mM phosphate buffer with 0.5 M NaCl (pH 7.5) using a Waring blender. The mixture was stirred for 30 min in an ice bath. The slurry was then passed through three layers of cheesecloth and centrifuged at 12,000 g for 30 min to separate the insoluble material. The supernatant was collected and adjusted to 20% saturation (NH₄)₂SO₄ (m/m), allowed to stand at 4 °C for 3 h without the precipitate, and combined with (NH₄)₂SO₄ to a final concentration of 60% saturation. After 3 h, the supernatant was centrifuged at 12,000 g for 30 min, and the water-insoluble pellet was redissolved in 50 mM PBS (pH 7.5) and extensively dialyzed against the same buffer thrice. This fraction was filtered through a 0.22 µm membrane and then loaded onto a DEAE-Sepharose Fast Flow column. The column (1.6 cm × 15 cm) was equilibrated with 50 mM PBS (pH 7.5). The sample was eluted using a gradient of 0–0.6 M NaCl in 50 mM phosphate buffer (pH 7.5) in a total volume of 350 mL using an ÄKTA pure system at a flow rate of 1.0 mL/min and absorbance at 280 nm. Fractions containing 11S globulin were pooled and dialyzed with 50 mM PBS buffer containing 0.15 M NaCl (pH 7.5). The samples were concentrated and applied to a HiPrep16/60 Sephacryl S-200 HR eluted with 200 mL of the same dialysate buffer at 0.2 mL/min.

SDS-PAGE analysis

Protein was subjected to SDS-PAGE in accordance with the method described by Laemmli (1970) and Kasran (2013) under reducing and non-reducing conditions. The protein samples were reconstituted to 1 mg/mL. Aliquots containing 20 µg of the sample were loaded onto 12% separating gels, and electrophoresis was performed with a vertical electrophoresis system (GE Healthcare, Montréal, PQ, Canada) at 80 V/gel until the tracking dye migrated to the gel edge. The gels were stained with Coomassie Brilliant Blue R-250.

Protein identification by MALDI-TOF/TOF MS

The MALDI-TOF/TOF MS platform was described by Baldwin (2004) and Zhou (2010). In brief, JSG subunits were separated electrophoretically using a 12% SDS-PAGE gel. Subsequently, gel spots were hydrolyzed by trypsin. All mass spectra were obtained on an Autoflex speed™ MALDI-TOF-TOF (Bruker Daltonik GmbH, Germany) in positive ion mode at an accelerating voltage of 20 kV with a nitrogen laser of 355 nm, repetition rate of 200 HZ, and optimal quality resolution of

1500 Da. The signals were collected between 700–3200 Da. All mass spectra of the experimental samples were obtained in default mode. Data were obtained from the SWISS-PROT and NCBI databases by using the MASCOT search engine (<http://www.matrixscience.co.uk>) with one missed cleavage site for the identification of purified protein based on sequence homology.

Determination of amino acid content

The amino acid content of globulin was determined based on the Chinese Standard GB/T 5009.124-2003 (2004). In brief, JSG (100 mg) was hydrolyzed with 10 mL of 6 M HCl containing 10 mM phenol and 0.2% (v/v) 2-mercaptoethanol for 24 h at 110 ± 1 °C under a nitrogen atmosphere. The hydrolyzed product was analyzed using an automatic amino acid analyzer L-8900 (Hitachi, Tokyo, Japan). Tryptophan content was determined by HPLC after conducting hydrolysis, as described by Yust (2004). The amino acid score (AAS), total EAA, total non-EAA, and total amino acid (TAA) were calculated according to the method reported by Yu et al. (2014).

Thermal property analysis

The thermal properties of JSG were measured using a differential scanning calorimeter (DSC 204 F1, Netzsch, Germany) in accordance with the method reported by Li-Chan (2002). The protein sample (1 mg) was placed in an aluminum pan, and 10 μ L of 10 mM phosphate buffer (pH 7.5) was added. The pan was hermetically sealed at a heating rate of 10 °C/min from 20 °C to 250 °C. An empty sealed pan was used as reference. Denaturation temperature (T_d) and denaturation enthalpy were calculated from the thermogram using Proteus Version 4.2.1 (Netzsch, Germany).

Far-UV circular dichroism (CD) spectral analysis

Optically clear JSG solutions (0.25 mg/mL) were used to record CD spectra (190–260 nm) in a 1 mm quartz cuvette with a MOS-450 Spectrometer (Bio-Logic). CD spectra were obtained in 1 nm wavelength steps and an acquisition duration of 5.0 s at 25 °C under constant nitrogen purge. CD data were expressed in terms of mean residue ellipticity. Data on secondary structure were interpreted by visual assessment of the spectra and by using the computer program CDPro.

Intrinsic fluorescence spectral analysis

Optically clear JSG solutions (200 μ g/mL) in 20 mM phosphate buffer (pH 7.5) for fluorescence spectra were determined using a Hitachi fluorescence spectrometer (F-4500, Hitachi Ltd., Japan). The protein was excited at 280 nm, emission wavelength spectra ranged from 290 nm to 450 nm at 25 °C, and each emission slit was set at 5 nm in a 1 cm quartz cuvette. The protein solutions were incubated overnight at 25 °C with urea (1–8 M) or guanidine hydrochloride (1–6 M) for 2 h at pH 2–13 and 25 °C before fluorescence spectral analysis.

RESULTS AND DISCUSSION

Purification of JSG

The elution profile with 0–0.6 M NaCl is shown in Figure 1A, anion-exchange chromatography showed four peaks, the relative molecular weight of all the fraction were presented by analysis of SDS-PAGE in our previous work (Wang et al, 2015). Peak 2, which was rich in 11S globulin, was identified by SDS-PAGE (SUN, 2015). Subsequently, the DEAE fraction rich in 11S globulin was pooled, concentrated to 20 mg/mL, and then filtered through a 0.22 μ m membrane for further purification on a HiPrep16/60 Sephacryl S-200 HR column (Figure 1B). Two fractions were obtained, and peak A was the desired fraction after identification by SDS-PAGE.

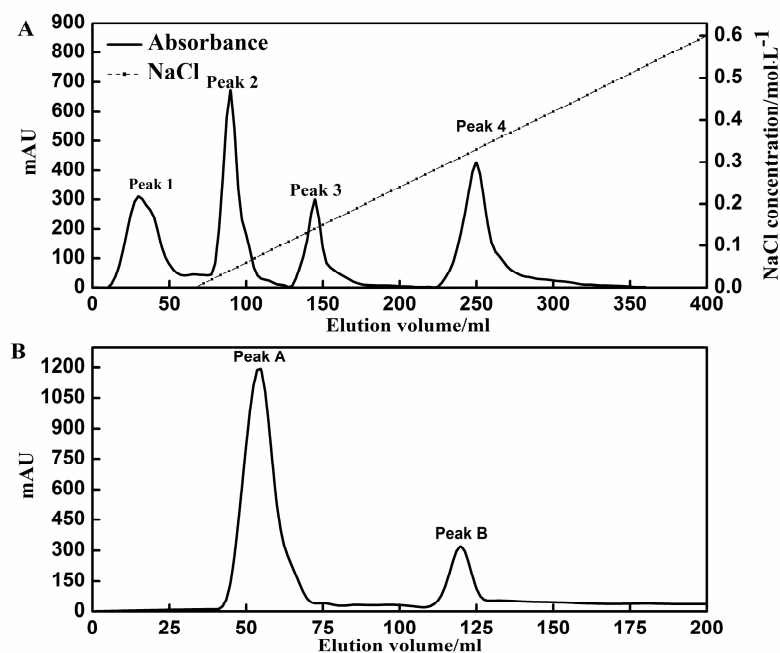


Figure 1: Purification of JSG. (A) Anion-exchange chromatographic of JMM seed protein. (B) Gel filtration chromatography of JMM seed protein.

PAGE analysis

The first gel filtration chromatography fraction containing the target protein was examined through 12% reducing and non-reducing SDS-PAGE (Figure 2). In the absence of a reducing agent (β -mercaptoethanol), JSG showed a single protein band in the region of 40 kDa. Under reducing conditions, the molecular weight (MW) of the bands in JSG appeared at 23 and 20 kDa. These results indicate that JSG contained disulfide bonds that held the polypeptides together (Orruno and Morgan 2007). This feature is typical among 11S globulins from other nuts, such as coconut, which yielded 34 and 24 kDa subunits on the SDS-PAGE (Garcia et al. 2005). Similarly, the 11S globulin from *Wrightia tinctoria* showed a major band of ~56 kDa under non-reducing conditions and two major polypeptides of approximately 32–34 kDa and 22–26 kDa under reducing conditions (Kumar et al. 2013). Nearly identical band patterns and values were also obtained for the 11S globulin from hazelnut seed (Rigby et al. 2008). These reports suggested that JSG likely belongs to the 11S globulin family.

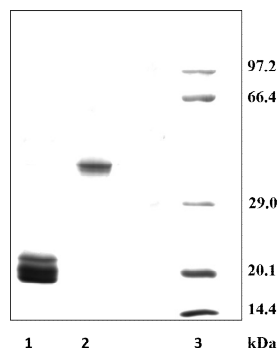


Figure 2: SDS-PAGE analysis of JSG. Lane 1, purified protein in the presence of a reducing agent; lane 2, purified protein in the absence of a reducing agent; lane 3, molecular weight marker.

Protein identification

For further protein identification, the subunits with MW ~23 and ~20 kDa were analyzed by MALDI-TOF/TOF (Figure 3). The results showed that the ~23 kDa subunit had six major mass ion peaks

of 1256.625, 1539.708, 1700.828, 2123.994, 2463.131, and 2987.5001 m/z ; whereas the other secondary peaks contained 933.551 and 2782.230 m/z . Similarly, strong peptide ions of the ~20 kDa subunit were 1373.773, 1667.953, 1859.946, and 2632.43 m/z with relatively weak peaks at 1002.565, 2062.968, and 2298.178 m/z . In accordance with the above description, the ~23 kDa subunit was considered distinct from the ~20 kDa subunit, and the two subunits exhibited different amino acid sequences. The accession number for *J. regia* 11S globulin in Table 1 was the same for both ~23 and ~20 kDa subunit matching sequences and the Mascot search results of Juglandaceae are listed in Table 1. Among the matches containing these sequences, the ~23 kDa subunit matched the 11S globulin of *J. regia* with a score of 74 and the 11S legumin of *Carya illinoensis* with a score of 52 (protein scores greater than 39 were considered significant). Moreover, the ~20 kDa subunit was similar to the 11S globulin of *J. regia* with a score of 54. The above data suggested that JSG was a member of the 11S globulin protein family.

Evaluation of amino acid

The amino acid content and composition of proteins greatly influence their structures and functions (Sharma et al. 2010). The amino acid composition of JSG and soy protein isolate (SPI) were compared (Table 2). The EAA recommended by FAO/WHO (2007) are also listed in Table 2 to serve as reference. JSG contains high acidic amino acids (25.59%) and hydrophobic amino acids (23.89%). These characteristics provide JSG with its strong hydrophobicity and non-covalent interactions, such as ionic bonding (Orruno et al. 2007). Weakening the non-covalent interaction and strengthening the repulsive force of electric charges between globulin molecules under the effect of NaCl reduces coherence and enhances solubility (Mazzafera et al. 2010).

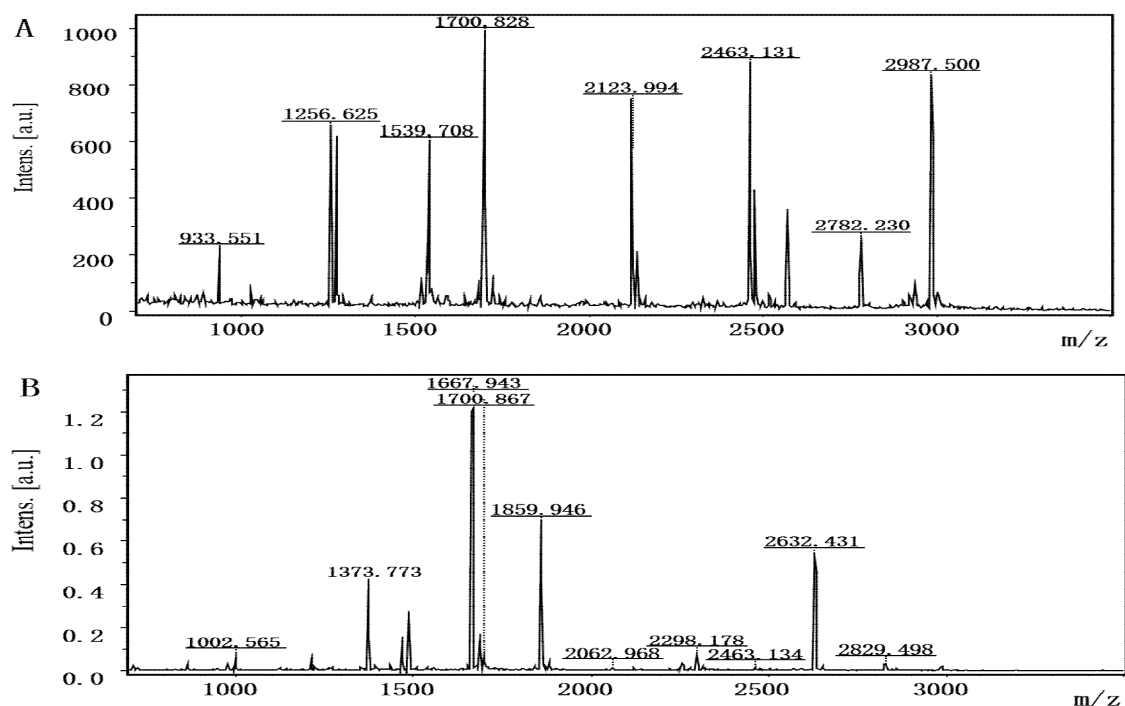


Figure 3: Tryptic analysis of the ~23 kDa (A) and ~20 kDa (B) gel bands of 11S JSG by MALDI-TOF/TOF MS.

Table 1 Amino acid sequence of reported *Juglans regia* peptides from the PMF of the ~23 and ~20 kDa subunit from the 11S globulin protein of walnut with the MASCOT database globulin protein of walnut with the MASCOT database

Band no.	Protein	Accession no. ^b	MW/pI ^c	Sequence coverage (%) ^d	Score ^e	Peptide hit	Peptides identified
23kDa	seed storage protein of <i>Juglans regia</i>	gi 56788031	58394/6.80	16	74	4	R.QQQQFGQQQLNR.L R.IEAEAGVIESWDPNQIQQFQ CAGVA VVR.R R.GITGVLPFGCPETFEESQR.Q R.NFYLAGNPDDEFAPQGGQEQEQR.R
	11S legumin protein of <i>Carya illinoensis</i>	gi 158998782	58307/6.46	8	52	2	R.GITGVLPFGCPETFEESQR.Q R.NFYLAGNPDDEFAPQGGQEQEQR.R
20kDa	seed storage protein of <i>Juglans regia</i>	gi 56788031	58394/6.80	25	54	8	R.WLQSLAER.G R.ADVITEAGRI K.TNENAMVSLAGR.T R.ISTVNSHTLPVLR.W R.ALPEEVLATAFQIPRE R.GITGVLPFGCPETFEESQR.Q R.GALYSDALVYFNWNLNANSVVYALR.G R.IEAEAGVIESWDPNQIQQFQ CAGVA VVR.R

a. Accession number in the NCBI database.

b. MW, molecular weight; pI, isoelectric point.

c. Amino acid sequence coverage of the protein with respect to matched tryptic digest fragments.

d. Each protein score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event; a value over 39 ($p < 0.05$) indicates a statistically significant match. *Peptide mass fingerprinting was performed using the Mascot search program, interrogating the NCBI database with a mass tolerance of 50 ppm.

Table 2 Amino acid content (%) of JSG (mg/100 mg dry powder)

Amino acid	JSG	SPI	FAO/WHO		Amino acid scores (%) ^e	
			Child	Adult	JSG	SPI
Asp ^{b,c}	8.91	8.90				
Thr ^a	3.43	2.81	3.40	0.90	100.88	82.65
Ser ^b	5.23	3.84				
Glu ^{b,c}	16.68	16.97				
Gly ^{b,c}	3.94	4.31				
Ala ^b	2.70	3.56				
Val ^a	4.11	3.63	3.50	1.30	117.43	103.71
Met ^{a,c}	1.39	0.92	2.70	1.70	51.48	34.07
Ile ^a	3.56	3.02	2.80	1.30	127.14	107.86
Leu ^{a,c}	4.78	5.76	6.60	1.90	72.42	82.27
Tyr ^c	3.47	3.21				
Phe ^{a,c}	2.21	4.32	6.30	1.90	35.08	68.57
His	1.47	2.30	1.90	1.60	77.36	121.05
Lys ^{a,c}	1.73	4.79	5.80	1.60	29.82	82.59
Arg ^c	10.36	6.90				
Pro ^b	2.89	3.14				
Trp ^{a,c}	1.95	1.01	1.10	0.50	177.27	91.82
TEAA	23.16	26.26				
TNEAA ^d	55.65	53.13				
TAA	78.81	79.39				
TEAA/TAA	0.29	0.33				
TEAA/TNEAA	0.42	0.49				

a. Essential amino acid; b. Flavour amino acid; c. Medicinal amino acid; d. Total non-essential amino acid.

e. Amino acid scores (%) of JSG or SPI compared to the FAO/WHO pattern for (2–5 years old) child.

JSG and SPI contain different types of amino acids. Protein sensory properties good acceptance can be explained by its amino acids profile due to glutamic and aspartic acids which are responsible for stronger umami taste (Buchtov et al., 2015; Deng et al., 2015). JSG and SPI have similar amounts of Asp and Glu. JSG has high content of 25.59% (dry basis), which accounts for 32.47% of the TAA at 25 °C. Abundant arginine in JSG can promote secretion of pituitary and pancreatic hor-

mones, boost immunity, and inhibit tumor protein synthesis and growth (Yu et al. 2014). The EAAs in JSG accounted for 29.38% of the TAA. Phenylalanine content of JSG (2.21%) was lower than SPI (4.32%), and lysine content of JSG (1.73%) was also significantly less than SPI (4.79%); however, all other EAAs between JSG and SPI were similar. The EAA content in JSG was superior to the reference values recommended by FAO/WHO for adults (except for methionine and histidine), and the contents of threonine, valine, isoleucine, and tryptophan met the recommended values for children (Comai et al. 2007). Thus, an appropriate amount of methionine can be added to improve the biological function of JSG. Based on the AAS displayed in Table 2, the first limiting amino acid in JSG is lysine, which is common in many plant proteins (Zhou et al. 2010), and the second is phenylalanine. The balanced amino acid content and high arginine content of JSG endow high biological value to this protein. Therefore, JSG can be used as a food supplement.

Thermal properties

Thermal denaturation of proteins is closely related to changes in their conformational space, which may significantly affect physiological activities (Li-Chan and Ma 2002). A representative DSC thermogram of JSG showed a single endothermic peak (Figure 4). The T_d of JSG was 96.83 °C, which was within the reported T_d range (83.8 °C–107.8 °C) for seed globulins (Marcone et al. 1998). Lentil proteins possess higher thermal stability than common bean proteins because of their higher β -sheet content (Carbonaro et al. 2008). The ΔH of JSG was 12.07 J/g, which was higher than that of protein isolates from kidney beans and field peas (Shevkani et al. 2015). JSG may have a more ordered structure than the protein isolates from these plant sources. Also, the T_d for globulin powder was determined in our previous work, DSC spectrogram showed two endothermic peaks, the denaturation temperatures were 98.08 °C and 155.33 °C, respectively (WANG et al. 2015). Thus, the high T_d and ΔH of JSG suggest that this protein might not undergo denaturation during extraction and purification.

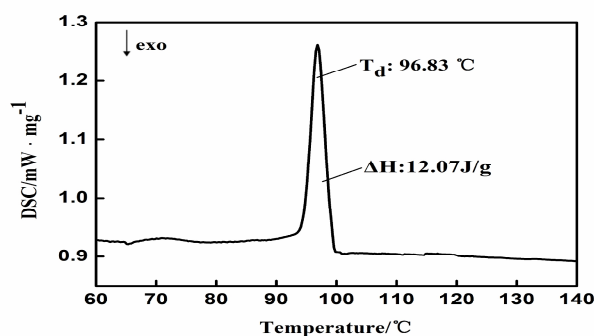


Figure 4: Thermal analysis chart of JSG.

Far-UV CD spectral analysis

CD is widely used to investigate conformations in protein solutions (Daniele et al. 2015). As displayed in Figure 5, the maximal negative mean residual ellipticities for JSG were observed near 210 nm, which was typical of 11S globulin proteins (Clara Sze et al. 2007; Kumar et al. 2013; Sharma et al. 2010). The contents of α -helices, β -sheets, β -turn, and random coil were calculated using the average of Selcon3, Continll, and Cdsstr. The calculated results are listed in Table 3. The three methods yielded similar results for the secondary structure of JSG; however, Continll yielded higher α -helices and lower β -sheets than the other two programs. Considering these averages, JSG was primarily composed of β -sheets with small amounts of α -helices. This result is consistent with the 11S proteins of Brazil nut and *W. tinctoria* (Kumar et al. 2013; Sharma et al. 2010). Previous research indicated that the high T_d of JSG might have resulted from its high β -sheet content (Clara Sze et al. 2007). The high content of β -sheets and the high T_d of JSG verified this speculation.

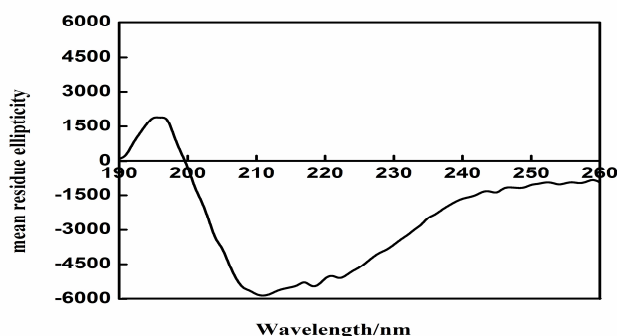


Figure 5: Far-UV circular dichroism analysis of JSG

Table 3 Secondary structure content of JSG

Calculation method	α -helices	β - sheets	β -turn	Random coil
Cdsstr	7.70	35.90	25.80	30.50
Continll	16.70	29.70	22.50	31.10
Selcon3	10.60	32.90	21.80	32.70
Average value	11.67	32.83	23.37	31.43

Intrinsic fluorescence spectral analysis

The fluorescence emission spectra of JSG are illustrated in Figure 6. The emission λ_{max} value for native JSG was 339. This emission profile is characteristic of tryptophan residues in a relatively hydrophobic environment, indicating that JSG is naturally folded (Dufour et al. 1994). Upon GdnHCl denaturation, the spectral peaks shifted toward longer wavelengths with a rapid decrease in intensity (Figure 6B). A similar result for 11S globulin from

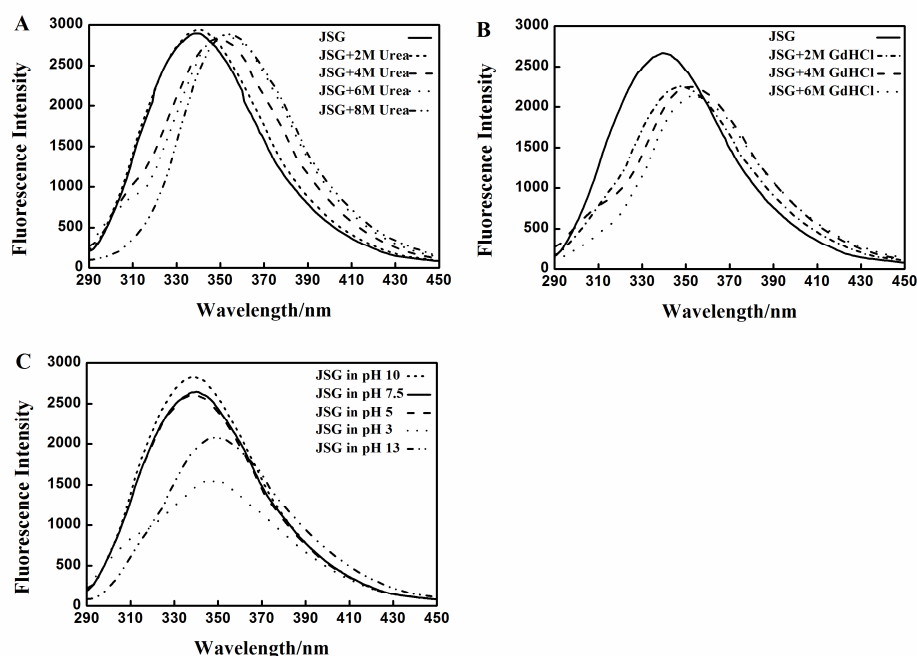


Figure 6: Fluorescence emission spectra of JSG. (A) Fluorescence spectra in the absence or presence of increasing concentration of urea. (B) Fluorescence spectra in the absence or presence of increasing concentration of GdnHCl. (C) Effect of pH on the intrinsic fluorescence of JSG

W. tinctoria in the presence of GdnHCl was previously reported (Kumar et al. 2013). Additional randomization of the 3D structure of JSG was altered, which caused changes in the local microenvironment of tryptophan residues and resulted in gradual tryptophan transition from the molecular in-

terior to the surface (Kumar et al. 2013). The effect of urea on JSG was similar to that of GdnHCl, but the change in fluorescence intensity was not evident (Figure 6A). This outcome indicates that GdnHCl was a stronger denaturant than urea. The emission fluorescence spectra of JSG at different pH values are displayed in Figure 6C. Spectral analysis indicated that the protein was stable at pH 5–10 and the peak intensity increased with increasing pH. When pH was greater than 10 or less than 5, the peak intensity was rapidly reduced with a pronounced redshift. A similar conclusion was reported by Kumar (2013).come indicates that GdnHCl was a stronger denaturant than urea. The emission fluorescence spectra of JSG at different pH values are displayed in Figure 6C. Spectral analysis indicated that the protein was stable at pH 5–10 and the peak intensity increased with increasing pH. When pH was greater than 10 or less than 5, the peak intensity was rapidly reduced with a pronounced redshift. A similar conclusion was reported by Kumar (2013).

CONCLUSIONS

JSG was extracted, purified, and structurally characterized as 11S globulin composed of two major polypeptides with molecular masses of 23 and 20 kDa. DSC spectral analysis of the protein showed high levels of alpha helices and β -sheets, whereas CD spectral analysis indicated that JSG is a relatively thermostable protein. The fluorescence spectra of JSG also showed a red shift under denaturing conditions and GdnHCl was a stronger denaturant than urea.

ACKNOWLEDGEMENTS

Funding for this work was provided by the National “863” Plan Project (NO. 2013AA102206) in China.

REFERENCES

- [1] Chinese Standard GB/T 5009.124-2003 (2004) Method for determination of amino acids in foods, China: Standards Press of China.
- [2] Baldwin, M.A. (2004). Protein Identification by Mass Spectrometry Issues to be Considered. *Mol Cell Proteomics*. 3, 1-9.
- [3] Bao, Y.H., Yu Y.Y. and Pan L.N. (2011). The Study on the Nutritive Components and Water-Soluble Protein Extraction of Juglans mandshurica Maxim Kernel. *Adv. Mater. Res.* 236, 1863-1866.
- [4] Buchtov, A.H., Dordevic, D., Kocarek, S. and Chomat, P. (2015). Analysis of chemical and sensory parameters in different kinds of escolar (*Lepidocybium flavobrunneum*) products. *Czech Journal of Food Sciences*, 33, 346-353.
- [5] Cai, L.H., Zeng, H.Y., Wang, Y.J., Liao, X.s. and Zhang, J.H. (2010). The Amino Acid Contents of Lotus Seed Peotein and its Nutritional Evaluation. *Acta. Nutrimenta Sinica*. 5, 503-506.
- [6] Carbonaro, M., Maselli, P., Dore, P. and Nucara, A. (2008). Application of Fourier transform infrared spectroscopy to legume seed flour analysis. *Food Chem.* 108, 361-368.
- [7] Chen, J., Liu, B., Ji, N., Zhou, J., Bian, H.j., Li, C.y., Chen, F. and Bao, J.k. (2009). A novel sialic acid-specific lectin from Phaseolus coccineus seeds with potent antineoplastic and anti-fungal activities. *Phytomedicine*. 16, 352-360.
- [8] Chen, Y.H., LI, J., Guo, X.X., QI, J.X., Wu, C.L. and Hao, Y.B. (2012). Research Advance of Antioxidation of Walnut. *Sci. Technol. Food Industry*. 17, 409-412.
- [9] Clara, K.W., Kshirsagar, H.H., Venkatachalam, M. and Sathe, S.K. 2007. A circular dichroism and fluorescence spectrometric assessment of effects of selected chemical denaturants on soybean (*Glycine max* L.) storage proteins glycinin (11S) and β -conglycinin (7S). *J. Agr. Food Chem.* 21, 8745-8753.

- [10] COMAI, S., BERTAZZO, A., BAILONI, L. (2007). The Content of Proteic and Nonproteic (Free and Protein-Bound) Tryptophan in Quinoa and Cereal Flours. *Food Chem.* 100, 1350-1355.
- [11] Coelho, M.B., Macedo, M.L.R., Marangoni, S., Silva, D.S.d., 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. Agr. Food Chem.* 5, 3050-3055.
- [12] DANIELE, T., CARLO, B. (2015). Induced circular dichroism as a tool to investigate the binding of drugs to carrier proteins: Classic approaches and new trends. *J. Pharmaceut Biomed*, 113, 34-42.
- [13] Deng, Y., Luo, Y., Wanga, Y. Zhao, Y. (2015). Effect of different drying methods on the myosin structure, amino acid composition, protein digestibility and volatile profile of squid fillets. *Food Chem*, 171, 168-176.
- [14] Dufour, E., Hoa, G.H.B. and Haertlé, T. (1994). High-pressure effects on β -lactoglobulin interactions with ligands studied by fluorescence. *Biochimica Et Biophysica Acta*. 1206, 166-172.
- [15] FAO/WHO (2007) Protein and amino acid requirements in human nutrition: Report of a Joint WHO/FAO. 2007. expert consultation, WHO technical report series no. 935, Place Published.
- [16] 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. Agr. Food Chem.* 53, 1734-1739.
- [17] 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 F: Structural Biology and Crystallization Communications*. 64, 641-644.
- [18] Kasran, M., Cui, S.W. and Goff, H.D. (2013). Emulsifying properties of soy whey protein isolate–fenugreek gum conjugates in oil-in-water emulsion model system. *Food Hydrocolloid*. 30, 691-697.
- [19] Kumar, P., N Patil, D., Chaudhary, A., Tomar, S., Yernool, D., Singh, N., Dasauni, P., Kundu, S. and Kumar, P. (2013). Purification and biophysical characterization of an 11S globulin from *Wrightia tinctoria* exhibiting hemagglutinating activity. *Protein Peptide lett*. 20, 499-509.
- [20] Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227, 680-685.
- [21] Li-Chan, E. and Ma, C.Y. 2002. Thermal analysis of flaxseed (*Linum usitatissimum*) proteins by differential scanning calorimetry. *Food Chem.* 77, 495-502.
- [22] Mandal, S. and Mandal, R. 2000. Seed storage proteins and approaches for improvement of their nutritional quality by genetic engineering. *Current Science-Bangalore*. 79, 576-589. (Chinese).
- [23] Marcone, M.F., Kakuda, Y. and Yada, R.Y. 1998. Salt-soluble seed globulins of various dicotyledonous and monocotyledonous plants—I. Isolation/purification and characterization. *Food Chem.* 62, 27-47.
- [24] Marsh, J., Rigby, N., Wellner, K., Reese, G., Knulst, A., Akkerdaas, J., van Ree, R., 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, S272-S285.
- [25] Martins, V.B. and Netto, F.M. 2006. Physicochemical and functional properties of soy protein isolate as a function of water activity and storage. *Food Researc. Int.* 39, 145-153.
- [26] Mujoo, R., Trinh, D.T. and Ng, P.K. (2003). Characterization of storage proteins in different soybean varieties and their relationship to tofu yield and texture. *Food Chem.* 82, 265-273.
- [27] Orruno, E. and Morgan, M. (2007). Purification and characterisation of the 7S globulin storage protein from sesame (*Sesamum indicum* L.). *Food Chem.* 3, 926-934.
- [28] Rassam, M. and Laing, W.A. (2006). The interaction of the 11S globulin-like protein of kiwi-fruit seeds with pepsin. *Plant Sci.* 6, 663-669.

- [29] Renkema, J., Knabben, J. and Van Vliet, T. (2001). Gel formation by β -conglycinin and glycinin and their mixtures. *Food Hydrocolloid*. 15, 407-414.
- [30] Rigby, N.M., Marsh, J., Sancho, A.I., Wellner, K., Akkerdaas, J., van Ree, R., Knulst, A., Fernández - Rivas, M., Brettlova, V. and Schilte, P.P. (2008). The purification and characterisation of allergenic hazelnut seed proteins. *Mol. Nutr. Food Res.* 52, S251-S261.
- [31] Sharma, G.M., Mundoma, C., Seavy, M., Roux, K.H. and Sathe, S.K. 2010. Purification and biochemical characterization of Brazil nut (*Bertholletia excelsa* L.) seed storage proteins. *J. Agr. Food Chem.* 9, 5714-5723.
- [32] Shevkani, K., Singh, N., Kaur, A. and Rana, J.C. 2015. Structural and functional characterization of kidney bean and field pea protein isolates: A comparative study. *Food Hydrocolloid*. 43, 679-689.
- [33] Shewry, P.R. and Halford, N.G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *J. Exp. Bot.* 53, 947-958.
- [34] Sun, L.L., Min, W.H., Liu, J.S., Li, H.M., Fang, L., Zhang, L. and Du, Y.P. (2014). Functional properties of the protein isolate and fractions prepared from *Juglans mandshurica* Maxim kernel in China. *J. Food Eng. Tech.* 1, 9.
- [35] Wang X.F., Min W.H., Zhu Y.M., Liu C.L., Wu D., Cui L.Y. and Liu J.S. (2015). Extraction and purification of globulin from *Juglans mandshurica* Maxim and characterization of its functional properties. *Modern Food Science and Technology*, 31: 234-241.
- [36] Wei, L.L. (2009). Study on Pharmacological Effects of *Julans mandshureca* Maxim. *Heilong Jiang Medicine Journal*. 4, 532-534.
- [37] Yu, H., Li, R., Liu, S., Xing, R.e., Chen, X. and Li, P. (2014). Amino acid composition and nutritional quality of gonad from jellyfish *Rhopilema esculentum*. *Biomedicine Preventive Nutrition*. 3, 399-402.
- [38] Yu, Y. (2012). The protein extracted and Antihypertensive peptides preparation from the kernel of *Juglans mandshurica* Maxim. Ph.D. Dissertation Northeast Forestry University
- [39] Yust, M., Penroche, J., Girón-Calle, J., Vioque, J., Millán, F. and Alaiz, M. (2004). Determination of tryptophan by high-performance liquid chromatography of alkaline hydrolysates with spectrophotometric detection. *Food Chem.* 2, 317-320.
- [40] Zhou, T., Li, Q.H., Zhang, J., Bai, Y.F. and Zhao, G.H. (2010). Purification and characterization of a new 11S globulin-like protein from grape (*Vitis vinifera* L.) seeds. *Eur. Food Res. Technol.* 5, 693-699.