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ISOLATION AND CHARACTERIZATION OF A SMALL MOLECULAR WEIGHT PROTEIN OF LINSEED MEAL

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Key Word Index—*Linum usitatissimum*; Linaceae; linseed meal; small M_r protein; circular dichroism; isolation.

Abstract—A small M_r protein from linseed meal has been isolated by CM-Sephadex chromatography. The protein was found to be homogeneous by the techniques of gel filtration, polyacrylamide gel electrophoresis and ultracentrifugation. It had $S_{20,w}$ value of 1.6S. Amino acid composition of the protein revealed a high amount of glutamic acid, cystine, arginine and glycine. The absorption spectrum of the protein consisted of a peak at 280 nm with a shoulder at 290 nm. The fluorescence emission maximum was at 340 nm. The protein contained large amounts of α-helix and β-structure. SDS-PAGE showed the protein to consist of a single polypeptide chain. The M_r estimated by Archibald's method, sedimentation-diffusion method and gel filtration was 17 000, 16 000 and 15 000 respectively. Difference spectra studies as a function of pH and temperature showed no variation in the conformation of the protein, probably due to disulphide bridges.

INTRODUCTION

Albumins are widely distributed in the seeds of diverse species and they are a distinct class of seed proteins. They are water soluble, normally constitute 20 to 60% of the total proteins, depending on the seed and they remain in the supernatant fluid during the precipitation of globulins from the meal extracts. They require relatively large amounts of ammonium sulphate for precipitation by classical salting-out method, being relatively small M_r proteins. This fraction contains proteins having some biological activity like trypsin inhibitor or proteases. The published reports on the nature of small M_r proteins of seeds, with special reference to oilseed proteins, are limited. Schwenke *et al.* [1, 2] studied the albumins of sunflower and rapeseed, and Youle and Huang [3] reported the nature of small M_r proteins from various oilseeds, such as sunflower, mustard, peanut, cottonseed, castor bean, linseed, etc. However, most of the workers studied the small M_r proteins as a group.

Sedimentation velocity pattern of linseed proteins showed the presence of four components with $S_{20,w}$ values of 1.4, 5.0, 9.0 and 14.0 and the relative proportion of these were 20, 10, 66 and 4% respectively [4]. In this paper, we report the isolation of the major small M_r protein in a homogeneous form. Its physico-chemical characteristics were compared with those of the globulin fraction of linseed meal.

RESULTS AND DISCUSSION

The small M_r proteins of linseed are water soluble and are basic in nature [4]. The major small M_r protein was isolated by CM-Sephadex chromatography and the protein eluted with 0.09 M NaCl.

The homogeneity of the isolated protein was tested by gel filtration, PAGE and ultracentrifugation. In gel filtration, the protein gave a single symmetrical peak. PAGE at pH 4.5 and 3.5 showed a single band. The protein corresponded to the major band of water soluble proteins. Sedimentation velocity experiment gave a single peak with $S_{20,w}$ value of 1.6S. Thus, the protein appeared to be homogeneous by the above physico-chemical techniques.

The protein contained no phosphorus and had less than 0.5% carbohydrate. The amino acid composition of the protein (Table 1) is characterized by a large amount of glutamic acid (35.0 g/16 g N), lysine (4.9 g/16 g N), arginine (13.1 g/16 g N), cysteine (3.5 g/16 g N) and glycine (8.3 g/16 g N) and small amounts of histidine, tyrosine and aspartic acid compared to the large M_r (12S) protein [5]. The large amount of ammonia liberated after hydrolysis of the protein suggests the presence of aspartic and glutamic acids in amidated form. Hence, the protein is basic in nature. A large amount of glutamic acid and cysteine were reported in the small M_r proteins of oilseeds and it was suggested that the sulphur amino acids act as a reservoir during the seed germination [2, 3]. Though it was suggested that the small M_r proteins might contain trypsin inhibitor or proteases, we did not observe such activities in the 1.6S protein.

The absorption spectrum consisted of the maximum at 280 nm and a shoulder at 290 nm. The fluorescence emission maximum was at 340 nm when excited at 280 nm. The shoulder at 290 nm in the absorption spectrum and the fluorescence emission maximum at 340 nm could be due to the tryptophan residues in the polar environment of the protein [6]. The free tryptophan content estimated by NBS [7] showed that 76% of the total tryptophan residues were in the polar environment.

The near UV-CD spectrum in the range 250 to 330 nm showed a broad peak from 255 to 285 nm and a shoulder at 290 nm. The peak in the broad range of 30 nm (255 to 285 nm) may be due to disulphide bridges [8]. The protein contained 3.5 g/16 g N cysteine as revealed by the amino acid analysis. The peak at 290 nm may be due to tryptophan in the polar environment of the protein [6].

Table 1. Amino acid composition of small M_r protein of linseed meal*

Amino acid	g/16 g N	Number of residues per mole protein†
Asp‡	5.5	8
Thr§	2.1	4
Ser§	3.9	8
Glu‖	35.0	45
Pro	3.0	5
Gly	8.3	25
Ala	1.9	5
Val	2.6	4
½Cys§	3.5	3
Met	0.8	1
Ile	2.8	4
Leu	5.4	8
Tyr	1.4	1
Phe	2.4	3
Lys	4.9	7
His	1.6	2
Arg	13.1	14
Try¶	2.0	2

*Average of duplicate determinations.

†An M_r of 17000 determined by Archibald method was used in calculations.

‡Includes asparagine.

§Values are at zero hr of hydrolysis by extrapolating 24, 48 and 72 hr of hydrolysis values.

‖Includes glutamine.

¶Estimated by the method of ref. [7].

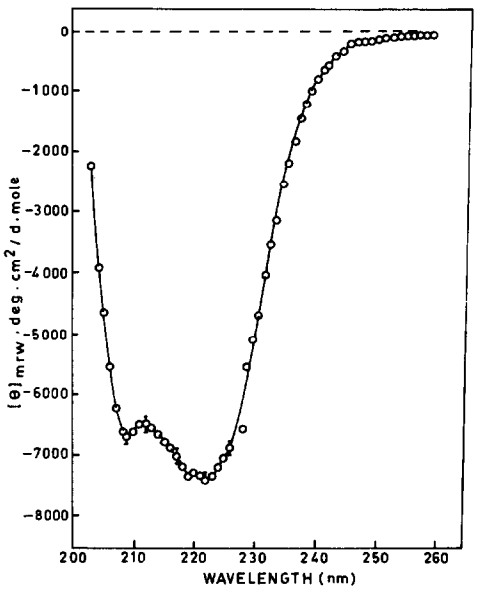

Fig. 1. Far UV-CD spectrum of small molecular weight protein in 0.5 M NaCl.

Far UV-CD spectrum in the range 200 to 260 nm exhibited two minima at 222 nm and 209 nm (Fig. 1). The computed helical content by the method of Greenfield and Fasman [9] and Chen and Yang [10] was 26% and 32% respectively, and the β-structure content calculated by the method of Sarkar and Doty [11] was 51%. The infrared spectrum recorded in the range of 1600–1700 cm^{-1} exhibited strong bands at 1635, 1645 and 1650 cm^{-1} which may be due to anti-parallel β-structure and aperiodic and helical regions respectively in the secondary structure of the protein [12, 13]. From the above spectra, the protein appears to belong to $\alpha+\beta$ class of proteins resembling the characteristics of typical α-helical organization of polypeptide back-bone described by Manavalan and Johnson [14]. A highly organized structure of small M_r proteins (albumins) has been reported in sunflower and rapeseed [1, 2].

End group amino acid analysis of the protein was done to study the subunit composition. The *N*-terminal amino acid was alanine, and *C*-terminal amino acid was lysine. SDS-PAGE of the protein exhibited a single sharp band, showing a M_r of 18000. The diffusion coefficient, $D_{20,w}$, of the protein was estimated to be 10.74×10^{-7} cm^2/sec. The M_r estimated by Archibald's method, sedimentation-diffusion and gel filtration was 17000, 16000 and 15000 respectively. The partial specific volume of the protein was assumed as 0.73. The variation in values obtained by SDS-PAGE and other physical methods is not significant and is within the limits of experimental error.

Difference spectra as a function of pH (in the range of 2–10) and temperature (in the range of 15–80°) showed that the protein is highly stable. Only at pH 10 was a red shift evidenced, probably due to ionization of tyrosyl residues. Similar observations of conformational stability of rapeseed and sunflower albumins as a function of pH, electrolyte concentration and temperature [1, 2] have been attributed to the stability of the disulphide bridges.

The major differences in the physico-chemical characteristics of the small M_r (1.6S) protein in comparison with the large M_r (12S) protein can be summarized as follows. The fluorescence emission maximum of 1.6S protein was at 340 nm, in contrast to 320 nm for 12S protein. The 1.6S protein consisted of a single polypeptide chain whereas the 12S protein showed five subunits on SDS-PAGE and six subunits on urea-PAGE. There was considerable difference in the secondary structure of these proteins. The small M_r protein exhibited a more ordered structure (α-helix and β-structure) in contrast to the high aperiodic structure of the large M_r protein. Also, the small M_r protein contained larger amounts of lysine, cysteine, glutamic acid and glycine and smaller amounts of methionine, aspartic acid, alanine, valine, phenylalanine and tyrosine than the large M_r protein. The 12S protein dissociated in the presence of low ionic strength buffers in contrast to the stability of 1.6S protein over a range of pH and temperature.

EXPERIMENTAL

Defatted linseed meal was prepared according to ref. [4]. CM-Sephadex C-50 and Sephadex G-100 were from Pharmacia and standard DNS-amino acids and M_r markers were from Serva and Sigma respectively. Carboxypeptidase-A was from Boehringer.

Isolation of small M_r *(1.6S) protein.* Defatted linseed meal (20 g) was stirred with 300 ml H_2O for 1 hr at ambient temperature and centrifuged at 1160 *g* for 10 min; the supernatant was dialysed

against water and the water solubles were lyophilized. The lyophilized protein (200 mg) was dissolved in 5 ml of 0.01 M NaPi buffer, pH 7, dialysed against the same buffer overnight and loaded on a CM-Sephadex column (2.2 × 20 cm) which had been equilibrated with the NaPi buffer. The unadsorbed proteins were eluted with the buffer until there was no UV absorbance of the eluant. The adsorbed proteins were eluted with a linear NaCl gradient in the range, 0–0.4 M NaCl in 0.01 M NaPi buffer, pH 7. The fractions corresponding to the peak eluting at 0.09 M NaCl were pooled, dialysed against H_2O and lyophilized. All measurements were made in 0.5 M NaCl.

Gel filtration. Sephadex G-100 that had been equilibrated with 0.5 M NaCl was packed into a column (1.8 × 86 cm). The protein (30 mg) was dissolved in 0.5 M NaCl, loaded on the column and eluted with 0.5 M NaCl at 21 ml/hr. Fractions of 2 ml were collected and the *A* was monitored at 280 nm.

Polyacrylamide gel electrophoresis (PAGE) was performed in 7.5% gels in 0.025 M NaOAc buffer, pH 3.5 or 0.02 M/3-alanine–acetate buffer, pH 4.5. Gels were pre-run at 1 mA/tube and the total duration of the experiment was 4 hr with 6 cm gels.

Ultracentrifugation. The sedimentation velocity experiment was performed with 1% protein soln in 0.5 M NaCl at 27° at 68 000 rpm in a Spinco Model E analytical ultracentrifuge equipped with rotor temp indicating and control (RTIC) system and phase plate Schlieren optics. $S_{20,w}$ values were calculated from the photographs by the standard procedure [15].

Absorption and fluorescence spectra were recorded in a Perkin–Elmer double beam recording spectrophotometer and Perkin–Elmer spectrofluorimeter respectively at 28°.

Carbohydrate and phosphorus content. Total carbohydrate and phosphorus content of 1.6S protein was estimated by the method of refs [16, 17] respectively.

Circular dichroism. These measurements were made in a JASCO-J 20C automatic recording spectropolarimeter at room temp (~ 28°). The instrument was calibrated with d-10 camphor sulphonic acid and the slits were programmed to yield 1 nm band width at each wave length. Near UV-CD measurements were made with 1 cm path length cells in the range 250–330 nm. Far UV-CD measurements were made with 0.5 or 1 mm path length cells in the range 200 to 250 nm. Mean residue ellipticity ($[\theta]_{MRW}$) values were calculated using the equations of ref. [18]. The α-helix content was calculated by the procedures of refs [9, 10], and the β-structure was estimated according to the method of ref. [11].

Infrared spectrum was recorded in the range 1500 to 1800 cm^{-1} in a JASCO DS-701G IR spectrophotometer at 20°. Cells of polyethylene sandwich system of 0.05 mm path length were used. The sample was dissolved in D_2O (7 mg/ml) containing 0.5 M NaCl.

M_r *determination.* (i) The diffusion coefficient was determined by a synthetic boundary experiment at 6800 rpm using a capillary cell centrepiece with an analytical ultracentrifuge as detailed above. $D_{20,w}$ values were calculated according to ref. [19]. (ii) M_r (Archibald method) was determined as described in ref. [20]. The speed used was 17 000 rpm. (iii) M_r by gel filtration was done on Sephadex G-100 according to ref. [21]. The gel was packed into a column (1.8 × 86 cm), equilibrated with 0.5 M NaCl and 30 mg sample or marker proteins were loaded. Fractions of 2 ml were collected and the *A* of the proteins was monitored at their maxima. A calibration curve was constructed with BSA, ovalbumin, soybean trypsin inhibitor, ribonuclease and cytochrome *c*. (iv) SDS-PAGE was performed in 15% separating gels by the method of ref. [22] in Tris–HCl buffer. Pepsin, trypsin, soybean trypsin inhibitor, myoglobin, lysozyme and cytochrome *c* were used as markers.

Amino acid analysis was carried out with a Yanagimoto LC-5S automatic amino acid analyser. The protein was hydrolysed at 110° with 6 M HCl containing 0.1% phenol and 0.01% β-mercaptoethanol *in vacuo* by the method of ref. [23]. Tryptophan was determined by the method of ref. [7]. The values are calculated by averaging the duplicate determinations. *N*-Terminal amino acid analysis was carried out by the dansyl chloride procedure [24] and *C*-terminal analysis was done with carboxypeptidase A enzyme according to ref. [25].

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