The seed proteins of linseed (*Linum usitatissimum* L.)

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**Abstract.** Linseed (*Linum usitatissimum* L.) was shown to contain a major globulin protein of mol. wt. 320 ± 20 (subunit mol. wt. 55, 54.5, 50, 45, 43, and 41). On reduction, the major globulin polypeptides cleaved into acidic subunits with mol. wt. ranging from 40 to 25 and basic subunits with mol. wt. ranging from 22 to 18. pi-values of the acidic subunits are between 4.5 and 6.5, and basic subunits are between 7.0 and 8.0. No subunits contained covalently bound carbohydrate. Other proteins were identified in linseed: the albumin-like proteins include a major albumin with a subunit mol. wt. of 25 and a low mol. wt. albumin with a subunit mol. wt. of 11. Albumin proteins had no covalently bound carbohydrate and no enzyme or inhibitor activities.

**Keywords:** Acidic subunits; Albumin-like proteins; Basic subunits; Legumin-like proteins; *Linum usitatissimum*.

**Introduction**

Despite the importance of linseed (*Linum usitatissimum* L.) as a source of oilcake and meal containing high levels of valuable proteins for animal consumption (Lennerts, 1983; Hatje, 1989), no work had been done on the characterization of its major seed proteins in relation to those of other oilseeds species such as *Glycine max* (Darper and Catsimpoolas, 1978; Thanh and Shibasaki, 1978; Koshiyama, 1983). This paper describes work intended to provide a more thorough characterization of the major linseed proteins.

**Materials and Methods**

Linseed (*Linum usitatissimum* L. cultivar Viking) was obtained from Cooperative Liniere De Fontaine-Cany, France. The seeds were germinated in an incubator at 25 °C and exposed to alternating 12-h periods of fluorescent illumination and darkness. Samples were taken every 12 hours, immediately frozen using liquid nitrogen, and lyophilized.

Sephadex G 100-120, Sephadex G 200-120, and concanavalin A were obtained from Sigma (London). Pea lectins and Phaseolin, *Pisum* legumin, and *Pisum* vicilin were obtained as a gift from the Biological Science Department, Durham University, Durham, England. All other chemicals were purchased from BDH Chemicals Ltd, U. K., and were of analytic grade.

**Preparation of total protein**

Flax seed was milled for 30 s in a Junk and Kunkle water-cooled mill. The meal was defatted with cold hexane (4 °C) three times; each time for 30 minutes. Total protein extract was prepared by extracting 30 mg of the defatted meal with 0.125 M Tris/borate buffer (pH 8.9) without the addition of 2-mercaptoethanol.

**Gel electrophoresis**

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out with gel slabs according to the method of Laemmli (1970); protein subunit bands were stained with Coomassie Blue R-250 by standard techniques. The gel slabs were calibrated with the following protein subunits as molecular-weight standards: transferrin (76), bovine serum albumin-BSA (67), albumin egg (ova albumin) (45), β-lactoglobulin (36.6), α-chymotrypsinogen-A (25.7), myoglobin (17.2) and cytochrome-c (12.7). 12% SDS-PAGE gels were used unless otherwise stated. Two-dimensional SDS-PAGE with nonreducing conditions for the first dimension and reducing conditions for the second dimension was carried out as described by Sammour et al. (1984).

**Mapping**

Isoelectric focusing in the first dimension and SDS in the second dimension were performed as described by Stegmann et al. (1988) and Laemmli (1970).

**Separation of globulins and albumins of linseed**

Total protein extracted with 0.05 M borax/borate buffer (pH 8.0) was extensively dialysed against 33 mM sodium acetate/acetic-acid buffer (pH 4.7). The precipitated globulins were separated from albumins by centrifugation at 23,000 g at 4 °C. The precipitate was resuspended in distilled water, then dialysed against 5 mM borax/borate buffer (pH 8.0) prior to freeze-drying.

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The supernatant liquid containing the albumins was extensively dialysed against distilled water and then freeze-dried.

**Purification of legumin-like linseed protein**

Freeze-dried globulins were dissolved in 0.1 M Tris/HCl buffer (pH 8.0) containing 0.25 M NaCl and 0.1% sodium azide as an antimicrobial agent, and applied to a column (2.6 cm. diam., 320 ml vol.) of Sephadex G-200 equilibrated and extensively washed with 0.1 M Tris/HCl (pH 8.0) and 0.25 M NaCl. The column was eluted by upward flow at the rate of 21 ml/hour.

**Purification of albumin-like protein of linseed**

Freeze-dried albumins were fractionated on Sephadex G-100 column equilibrated and run under the same protocol used for the purification of Legumin-like protein.

**Molecular weight determination**

A column of Sephadex G-200 (Sigma) 75 cm x 2.2 cm, in 0.1 M Tris/HCl (pH 8.0) containing 0.25 M sodium chloride and 0.05% (W/V) sodium azide was calibrated with the following mol. wt. markers: *Pisum* legumin (400), *Pisum* vicilin (150), phosphorylase B (98), transferrin (76), albumin egg (ova albumin) (45), myoglobin (17.2). The retention volume of each marker was determined (V_e). The void volume of the column (V_o) was also determined by eluting blue dextran (1000) on the column. V_e/V_o was plotted for each marker against its log mol. wt. Approximately 10 mg purified protein was applied to the column and eluted by upward flow at the rate of 8 ml/h.

**Carbohydrates determination**

Carbohydrates were determined by the phenol-sulfuric acid method (Dubois et al., 1956) or by reaction with thymol-sulfuric acid (Racusan, 1979) after SDS-PAGE. Phaseolin (the major storage protein of *Phaseolus vulgaris*) and bovine serum albumin run on the same gel slab were used as positive and negative controls.

**Amino acid analyses**

Protein samples were reduced and carboxymethylated with iodoacetamide (Bailey and Boulter, 1970) before acid hydrolysis, as described by Croy et al. (1980), and analysed on a Varian 5060 HPLC system, with a Micropack A. A. column (Varian; 15 cm x 4 mm diam.) using the Varian PCR-1 post-column derivatization and Fluorochrome detection system.

**Enzyme and inhibitor activities**

Enzyme and inhibitor assays (α- and β-D-glucosidases, β-D-galactosidases and β-D-mannosidase, amylase and amylose inhibitor, and trypsin inhibitor) were performed according to the procedures described by Sammour et al. (1984) and Croy et al. (1984).

**Haemagglutination assays**

Assays were performed according to the same protocol used by Croy et al. (1984); pea lectins and concanavalin A were used as standard haemagglutinins.

**Results**

**Analysis of total extractable proteins**

Mature dry flax seeds were extracted under two sets of conditions: 0.125 M Tris/borate buffer (pH 8.9) containing 2% SDS, with or without 2% 2-mercaptoethanol. The resulting extracts were analyzed by SDS-PAGE. The protein subunit patterns differed according to the presence or absence of 2-mercaptoethanol (Figure 1A). Since some subunits were only present if 2-mercaptoethanol was absent, and certain lower mol. wt. subunits only appeared in the presence of 2-mercaptoethanol, the presence of disulphide-bonded subunits was suspected. Two-dimensional gel electrophoresis techniques, with non-reducing conditions in the first dimension and reducing conditions in the second dimension, were used and showed that subunits of mol. wt. 55, 54.5, 50, 45, 43, and 41 were disulphide-linked subunits (Figure 1B).

![Figure 1](image.png)

*Figure 1.* A) SDS-PAGE of linseed proteins extracted with 0.125 M Tris/borate buffer, pH 8.9. Tracks 1–2: without addition of 2-mercaptoethanol; tracks 3–4: with addition of 2% 2-mercaptoethanol. B) Two-dimensional SDS-PAGE analysis of total linseed proteins. First dimension (horizontal) unreduced (-2ME); second dimension (vertical) reduced (+2ME). Scales indicate mol. wt. x 10^3.

The highest mol. wt. bands (55, 54.5, and 50) were cleaved with 2-mercaptoethanol into acidic subunits having mol. wt. about 40 (1A) and basic units with mol. wt. about 20 (1B) during the second dimension of electrophoresis. The other disulphide bonded bands formed two groups; acidic subunits (2A) with mol. wt. about 25 and basic subunits (2B) with mol. wt. around 20.
Proteins extracted by borax/borate buffer were fractionated into globulins and albums by dialysis against 33 mM sodium acetate buffer (pH 4.8). The precipitate (globulins) and supernatant liquid (albumins) were separated, lyophilized, and analysed by SDS-PAGE; the results are shown in Figure 2A. The major polypeptides, with mol. wt. of 55, 54.5, 50, 45, 43, and 41, were found in the globulin fraction, as were subunits with mol. wt. of 22.5 and 12.5. The albumin fraction was found to contain two bands, with mol. wt. of 25 and 11. On reduction with 2-mercaptoethanol the major polypeptides of the globulin proteins were cleaved into two groups of bands; acidic subunits with mol. wt. between 40 and 25, and acidic subunits with mol. wt. between 22 and 18 (Figure 2B).

Mapping was done for the globulins proteins, first by IEF, and then in the second dimension by SDS-PAGE. Isoelectric focusing of the component acidic and basic subunits of legumin subunit pairs was performed during second dimension gel analysis after separation of the subunits by isoelectric focusing in the first dimension (Figure 3). In all subunit pairs, the larger subunits have lower pl's, i.e. they are more acidic. All acidic subunits (A) fall in the pl range 4.5-6.5, and basic subunits (B) fall in the pl range 7.0-8.0. All subunits are highly heterogeneous.

Purification and properties of the major globulin proteins

The major globulin proteins of linseed were purified by precipitation from an extract of linseed, redissolution of the precipitate, and chromatography on Sephadex G-200. The elution profile had two peaks (Figure 4). The proteins precipitated in the first peak contain subunits of mol. wt. 55, 54.5, 50, 45, 43, and 41. This material separated into five bands during SDS-PAGE (Figure 5A), and into large subunits (α or acidic subunits) and small subunits (β or basic subunits) on analysis under reducing conditions (Figure 5B). The second peak separated into a number of low mol. wt. bands.
Molecular weight determination of linseed major globulins was carried out by gel filtration on a calibrated column of Sephadex G-200. The proteins were not resolved by this system, but ran as a major peak of mol. wt. 350 ± 20.

Carbohydrate residues covalently attached to the polypeptide chain were shown to be absent in all subunits, by treatment of the gels with phenol-sulfuric acid or thymol-sulfuric acid. Bovine serum albumin and phaseolin (the major storage protein of Phaseolus vulgaris) run on the same gel slab were used as positive and negative controls.

The amino acid composition of major linseed globulins (Table 1) shows high nitrogen content (glutamate/glutamine, aspartate/asparagine, and arginine), confirming its storage role (Derbyshire et al., 1976).

**Table 1.** Amino acid composition of legumin and albumin-like proteins (major and low molecular weight albumins)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Legumin</th>
<th>Albumin-like proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>10.06 ± 0.03</td>
<td>08.72 ± 0.05</td>
</tr>
<tr>
<td>Thr</td>
<td>06.08 ± 0.01</td>
<td>04.13 ± 0.03</td>
</tr>
<tr>
<td>Ser</td>
<td>07.99 ± 0.02</td>
<td>05.68 ± 0.04</td>
</tr>
<tr>
<td>GLU</td>
<td>17.13 ± 0.03</td>
<td>27.33 ± 0.15</td>
</tr>
<tr>
<td>Pro</td>
<td>02.05 ± 0.07</td>
<td>02.21 ± 0.02</td>
</tr>
<tr>
<td>Gly</td>
<td>09.80 ± 0.06</td>
<td>08.11 ± 0.06</td>
</tr>
<tr>
<td>Ala</td>
<td>03.79 ± 0.05</td>
<td>03.39 ± 0.04</td>
</tr>
<tr>
<td>Val</td>
<td>04.07 ± 0.05</td>
<td>04.13 ± 0.05</td>
</tr>
<tr>
<td>Cys</td>
<td>01.10 ± 0.03</td>
<td>00.36 ± 0.01</td>
</tr>
<tr>
<td>Met</td>
<td>02.01 ± 0.05</td>
<td>01.11 ± 0.03</td>
</tr>
<tr>
<td>Ile</td>
<td>04.13 ± 0.04</td>
<td>03.58 ± 0.06</td>
</tr>
<tr>
<td>Leu</td>
<td>06.59 ± 0.06</td>
<td>05.91 ± 0.05</td>
</tr>
<tr>
<td>Tyr</td>
<td>02.36 ± 0.07</td>
<td>02.21 ± 0.03</td>
</tr>
<tr>
<td>Phe</td>
<td>04.00 ± 0.06</td>
<td>03.12 ± 0.05</td>
</tr>
<tr>
<td>Lys</td>
<td>04.02 ± 0.03</td>
<td>05.02 ± 0.01</td>
</tr>
<tr>
<td>His</td>
<td>01.95 ± 0.02</td>
<td>02.08 ± 0.02</td>
</tr>
<tr>
<td>Arg</td>
<td>11.50 ± 0.08</td>
<td>11.14 ± 0.13</td>
</tr>
<tr>
<td>Trp</td>
<td>00.74 ± 0.01</td>
<td>00.74 ± 0.02</td>
</tr>
<tr>
<td>Amm</td>
<td>02.49 ± 0.07</td>
<td>01.58 ± 0.01</td>
</tr>
</tbody>
</table>

Each figure represents the mean of four amino acid analyses, plus or minus the standard deviations.

**Purification and properties of the albumin-like proteins**

The albumin-like proteins of linseed were purified by precipitation from an extract of linseed, redissolution of the precipitate, and chromatography on Sephadex G-100. The elution profile had two peaks (Figure 6). The electrophoresis pattern of the protein precipitated in the first peak shows two bands, with subunit mol. wt. of 25 kDa and 11 kDa. The second peak showed some artifacts (Figure 7).

Treatment of the electrophoresis gels with phenol-sulfuric acid or thymol-sulfuric acid showed that there were no carbohydrate residues covalently attached to the polypeptide chains in any subunits. Phaseolin (the major storage protein of Phaseolus vulgaris) and bovine serum albumin were run on the same gel slab as positive and negative controls.

The amino acids of linseed albumin-like (Table 1) proteins have a high nitrogen content (glutamate/glutamine, aspartate/asparagine, and arginine), suggesting its storage role in the seeds.

Albumin-like proteins were assayed for functional properties. They showed no activities in any of the enzyme assays (α- and β-D-glucosidases, β-D-galactosidases and β-D-mannosidase, and amylase). They exhibited no inhibition during amylase-inhibition assays with porcine pancreatic amylase or trypsin inhibition assays with bovine trypsin, and no haemaglutinating activity was associated with the albumin proteins.

**Figure 5.** A) SDS-PAGE of the purified legumin-like protein proteins eluted from Sephadex G-200 column. B) SDS-PAGE of the proteins eluted from Sephadex G-200 column and analysed reducing conditions. Track 1: total protein; track 2: globulin; tracks 3-4: protein precipitated in peak 1; track 5: protein precipitated in peak 2.

**Figure 6.** Chromatography of linseed albumin on Sephadex G-100 column; elution profile of the column.
Analysis of linseed material during germination

Seeds at different stages of germination were analysed by SDS-PAGE (Figure 8). The gel showed that globulin and albumin proteins degrade during germination, confirming their storage roles.

Discussion

The major linseed globulin protein is heterogeneous, as shown by gel electrophoresis, and consists of six polypeptides with mol. wt. 55, 54.5, 50, 45, 43, and 41. On the basis of the molecular weight of the protein, a hexameric structure seems likely, analogous to that proposed for the legumin of Pisum and Vicia faba (Wright and Boulter, 1974; Matta et al., 1981). After reduction with 2-mercaptoethanol, the six polypeptides cleaved into large subunits (α or acidic subunits) and small subunits (β or basic subunits). The acidic subunits are composed of two groups of subunits, one with mol. wt. between 40 and 38, and the other with mol. wt. of 25. The basic subunits have mol. wt. ranging between 22 and 18.

Further complexity is found in the heterogeneity of the major polypeptides, as shown by SDS-mapping of the major globulin combining isoelectric focusing in urea gel in the first dimension and SDS-PAGE in the second dimension. The data from isoelectric focusing indicate that linseed legumin shares other legumin-like proteins in the isoelectric points of the acidic and basic subunits (Krishan et al., 1979; Matta et al., 1981; Dalgalarmando et al., 1984; Robbellen et al., 1989). The data from isoelectric focusing in combination with the data from electrophoresis suggest the occurrence of considerable heterogeneity in linseed legumin.

This heterogeneity could be due to 1) single substitutions in amino acid sequences of polypeptides (these may be responsible for the charge heterogeneity of individual subunits); 2) more extensive differences in sequences among polypeptides, including differences in the total number of amino acids (these may be considered responsible for the size heterogeneity among subunits) (Matta et al., 1981); 3) the differential proteolysis of the proteins that can occur in vivo and after homogenization of the seed.

The amino acid composition of the major globulin protein of linseed contains higher levels of the sulphur amino acids (cystein and methionine) than is the average for other globulins proteins (Derbyshire et al., 1976; Youle and Huang, 1981). These data support the suggestion that the major globulin protein of linseed is a good candidate for investigating improving the nutritional quality of linseed.

The major globulin of linseed is clearly similar to the other legume globulins of Vicia faba and Pisum sativum legumin-type (mol. wt. 330,000-400,000 [11S], heterogeneous disulphide linked subunits containing non covalently-bound carbohydrate) (Wright and Boulter, 1974; Matta et al., 1981).
Other proteins, present in much smaller amounts, may be identified in linseed on the basis of the present results. From gel filtration on Sephadex G-100, the major (25kDa) and the low mol. wt. albumin (11kDa) are identified. Molecular weight determination on Sephadex G-200 indicated a mol. wt. of 50 for major albumin and 11 for low mol. wt. albumin. On the basis of the mol. wt., the subunit compositions of both the major and low mol. wt. albumin of linseed are analogous to that proposed for those of P. sativum (Croy et al., 1984).

The albums of linseed are clearly similar to P. sativum albumin because they contain non covalently-bound carbohydrate and are rich in sulfur amino acids (Croy et al., 1984). As reported by Croy et al. (1984), the major albumin is located in the cytoplasm, suggesting that this protein may have some metabolic or antimetabolic roles. The albumin-like proteins of the linseed, however, showed no activity in any of the enzyme assays, the albumin proteins exhibit no inhibition in amylase-inhibition assays with porcine pancreatic amylase or in trypsin inhibition assays with bovine trypsin, and no haemagglutinating activity was associated with these proteins. The degradation of the albumin protein on germination, and its high nitrogen content (glutamate/glutamine, aspartate/asparagine, and arginine) suggest its storage role.

In conclusion, the majority of linseed proteins are legumin-like proteins of mol. wt. 320 ± 20 and subunit mol. wt. of 55, 54.5, 50, 45, 43, and 41. On reduction with 2-mercaptoethanol, legumin-like protein polypeptides cleaved into acidic subunits with a mol. wt. between 25 and 40 and basic subunits with mol. wt. from 18 to 22. Legumin-like protein of linseed contains non-covalently bound carbohydrates and had pI-values between 4.5 and 8. Other proteins purified in this study were albumin-like and contained major (subunit mol. wt. 25) and low molecular weight albumin (subunit mol. wt 11). Neither major albumin nor low mol. wt albumin had metabolic or antimetabolic activity. Their degradation on germination and their richness in nitrogenous amino acids, however, suggest their storage role.

Literature Cited


亞麻 (Linum usitatissimum L.) 的種子蛋白

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本報告証實亞麻子含有一主要球蛋白，其分子量為 320±20 kDa，次單元分子量為 55，54.5，50，45，43 和 41 kDa。經還原後，此球蛋白的多肽鍵被切為分子量自 40 到 25 kDa 的酸性次單元和分子量自 22 到 18 kDa 的鹼性次單元。酸性次單元的 pI 值介於 4.5 和 6.5 之間，而鹼性次單元介於 7.0 和 8.0 之間。無次單元含塩鍵結合的醣類。其它亦被鑑定的種子蛋白有類似白蛋白的主要白蛋白，其次單元分子量為 25 kDa 和一低分子量的白蛋白，其次單元分子量為 11 kDa。白蛋白無塩鍵結合的醣類和無酶或抑制劑的活性。

關鍵詞: 酸性次單元；白蛋白；鹼性次單元；豆球蛋白；亞麻。