LUPIN SEED PROTEINS

1. A PHYSICO-CHEMICAL STUDY OF THE PROTEINS FROM BLUE LUPIN SEED (*LUPINUS ANGUSTIFOLIUS*)

by

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In South Africa, lupin is used in the improvement of soils which lack nitrogen and also as pasture for sheep. Very little is known about the proteins and the amino acid composition of the proteins of lupin seed. This paper, dealing with the physico-chemical properties of the globulins from blue lupin seed (*Lupinus angustifolius*), is the first outcome of a programme on lupin seed protein recently undertaken in this laboratory.

RITTHAUSEN[1] first studied and described under the name conglutin the characteristic proteid of the lupin seed. OSBORNE AND CAMPBELL[2] found that the globulin portion of blue and yellow lupin seed protein could be separated by fractional precipitation into two parts of different composition. DANIELSSON[3] has reported ultracentrifugal sedimentation diagrams for globulins of a large number of different species in the family Leguminosae, including three different lupins. Although DANIELSSON developed a method for separating pea globulins, he did not apply this to lupin globulins. The preparation and chemical modification of membranes from lupin seed globulins were described by PETRI AND STAVERMANN[4]. These writers separated the globulins into two fractions of different nitrogen content by OSBORNE AND CAMPBELL'S[2] method of precipitation with ammonium sulphate solutions of increasing concentration, but DANIELSSON[3] found that this method did not give pure protein fractions for the presumably closely similar pea globulins. No writer has discussed the question of removal of colour from lupin protein.

METHODS

Electrophoresis was carried out in a Tiselius electrophoretic apparatus manufactured by Messrs. Hilger Watts Ltd., using a medium U-tube equipped with a long centre section. Because of low protein solubility at 0–4° C, electrophoresis was carried out at 20° C, field strengths employed being limited accordingly[5]. Dialysis of protein solutions was routinely performed in a slowly rotating cellophane bag against a large volume of the required buffer solution. Dialysis was carried out for 24 hours with 2 or 3 changes of buffer.

Sedimentation studies were carried out at a protein concentration of *ca.* 1% in a Spinco electrically driven ultracentrifuge manufactured by Messrs. Specialised Instrument Corporation, Belmont, California. All the runs were performed at *ca.* 60,000 r.p.m., equivalent to a centrifugal force of *ca.* 250,000 × *g*.

Diffusion coefficients have been determined at 20° C by the free boundary method using the Tiselius U-tube for boundary formation. A plunger type of compensator was used to move the boundary between protein and buffer solution into the observation channel without disturbances.

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Preparation of proteins

Decorticated lupin seed was defatted by repeated extraction with pentane. The proteins of oil-free lupin seed meal were completely extracted with three separate volumes of 10% sodium chloride over a period of 24 hours at 4° C. The final saline extract was centrifuged at 2,500 r.p.m. and further cleaned by filtration through a pad of kieselguhr. The extracted proteins were precipitated by adding ammonium sulphate to 85% saturation, giving a preparation which was very yellow in colour. By prolonged dialysis some of the colour was removed, but the final solution was always yellow.

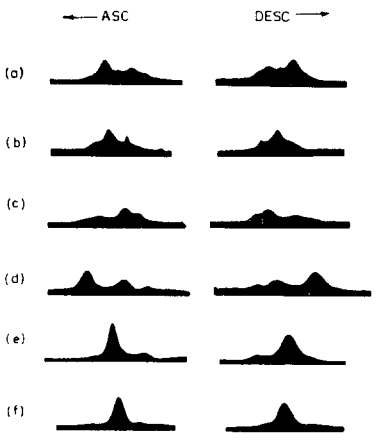

Fig. 1. Electrophoretic diagrams of lupin seed proteins.

Fig. 1(a) and 2(a) give respectively electrophoretic and sedimentation diagrams of this precipitate in borate buffer of ionic strength (I) = 0.2, pH = 8.8. Whereas only three components of sedimentation constants 11.6, 7.8 and 1.6 Svedberg Units (S.U.) (designated as the $s_{11.6}$, $s_{7.8}$ and $s_{1.6}$ components) were observed by sedimentation, electrophoresis showed four components.

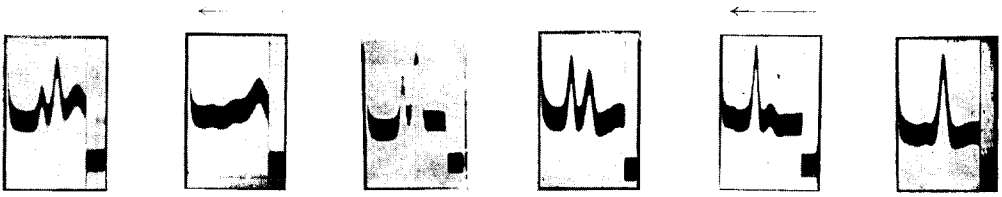

Fig. 2. Sedimentation diagrams of lupin seed proteins.

In the next experiment the total proteins of the 10% sodium chloride extract were separated into a fraction insoluble in water, *i.e.*, the globulin fraction, and a fraction soluble in water, *i.e.*, the albumin fraction. The albumin fraction, when examined in the ultracentrifuge, showed a major $s_{1.6}$ component and a small concentration of the $s_{7.8}$ component (Fig. 2(b)). In electrophoresis three components were observed (Fig. 1(b)). Fig. 1(c) and 2(c) show respectively electrophoretic and sedimentation diagrams of the globulin fraction. In electrophoresis three components were observed, whilst the sedimentation diagrams showed only the $s_{11.6}$ and $s_{7.8}$ components. From the sedimentation diagrams of Fig. 2(a) and 2(b), it would appear that the albumin fraction corresponds to a single protein, namely, the $s_{1.6}$ component in Fig. 2(a), and constitutes 20–25% of the total seed protein.

From the foregoing experiments it becomes clear that electrophoresis always showed one more component than observed by the ultracentrifuge. As all these preparations were yellow in colour, it was felt that this was due to interaction of proteins of lupin seed with coloured constituents occurring in the seed to form new components with different electrophoretic properties. It was therefore considered necessary to attempt to remove the colouring matter before further investigating the proteins present. Complete removal

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of these impurities under conditions such that the proteins were not denatured was achieved by the following method.

Defatted lupin seed meal was repeatedly extracted with 50–50 ethanol-water mixture at room temperature and then with cold water by using a Waring Blendor. The meal was next repeatedly washed with acetone at room temperature and left in air to dry. As 80% of the proteins present in the purified meal could be extracted with salt solution, it may be assumed that very little denaturation of the purified protein had occurred. The purified meal contains only the globulin constituents, as the albumin portion is removed in the water wash. Since the albumin fraction could not be freed from the yellow colouration, it was not further studied.

The proteins of purified lupin seed meal were extracted with 10% sodium chloride and then precipitated by adding ammonium sulphate to 85% saturation. Fig. 1(d) shows electrophoretic diagrams of this preparation in buffer $I = 0.1$, pH = 8.8, and Fig. 2(d) sedimentation diagrams in buffer $I = 0.31$, pH = 7.0. The sedimentation diagrams showed two major components ($s_{11.6}$ and $s_{7.8}$) and a small concentration of a slow component ($s_{1.6}$), and was thus in good agreement with Fig. 2(c) for the globulins before purification. The removal of colour did not therefore affect the sedimentation properties. As will be detailed shortly, the difference in relative proportions of the two components in Fig. 2(c) and 2(d) was due to a difference in the buffers employed. When, however, we compare the electrophoretic diagrams of Fig. 1(c) and 1(d), it is found that removal of colouring matter has had a profound effect. The peaks due to the various protein constituents in Fig. 1(d) are easily resolved and the number of peaks is in accord with the number of sedimenting components.

The fact that removal of colour does not much affect sedimentation velocities, but changes electrophoretic properties, seems to indicate that the amounts of colouring matter involved are small. Thus, they do not much affect the molecular weight or shape of the protein molecule to which they are attached, but may have a considerable influence on its electrical charge.

Fractionation of the two globulin components was carried out according to a slightly modified form of the procedure described by DANIELSSON[3] for the fractionation of pea globulins. The scheme of fractionation is outlined in Fig. 3.

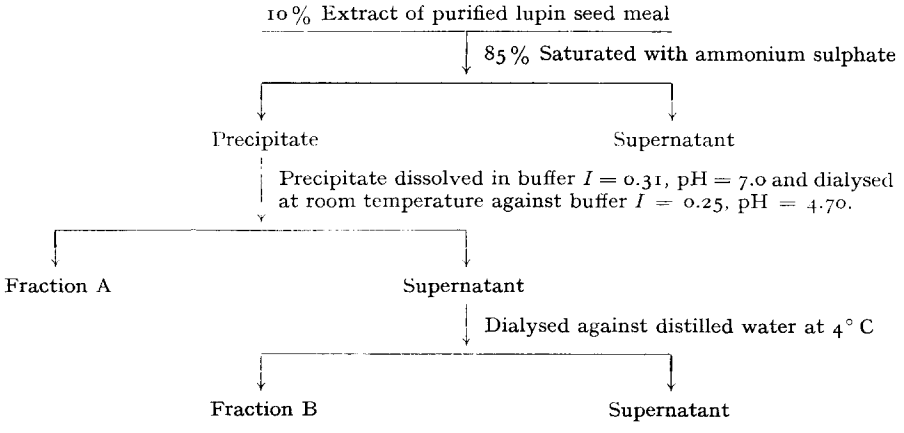

Fig. 3. Scheme of fractionation of lupin seed globulins.

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The proteins extracted from purified lupin seed meal were precipitated by adding ammonium sulphate to 85% saturation. The resulting precipitate was dissolved in phosphate buffer $I = 0.31$, pH = 7.0, centrifuged clear and then dialysed for *ca.* 24 hours at room temperature against an acetate buffer $I = 0.25$, pH = 4.70. The precipitate which formed during this dialysis was centrifuged off (fraction A) and the supernatant was dialysed against distilled water at 4° C and another precipitate (fraction B) formed.

Both fractions A and B were examined by electrophoresis in buffer $I = 0.1$, pH = 8.8 (Fig. 1(e), 1(f) and Table I) and by the ultracentrifuge in buffer $I = 0.31$, pH = 7.0 (Fig. 2(e) and 2(f)). The sedimentation diagram of fraction A contains a major $s_{11.6}$ component and a small concentration of the $s_{7.8}$ component and a similar electrophoretic diagram was obtained. For fraction B a single component ($s_{7.8}$) was observed in the sedimentation as well as the electrophoretic diagrams. The data of Table I indicate that the electrophoretic mobility of fraction A (the $s_{11.6}$ component) was higher than that of fraction B (the $s_{7.8}$ component).

TABLE I

ELECTROPHORETIC MOBILITY OF LUPIN SEED GLOBULINS AT 20° C, IN BUFFER $I = 0.1$, pH = 8.8

Preparation	*Mobility* × 10^{-4} $cm^2\ sec^{-1}\ volt^{-1}$			
	Descending		*Ascending*	
Total globulins	0.58	1.20	0.72	1.32
Fraction A ($S_{11.6}$)	—	1.16	—	1.33
Fraction B ($S_{7.8}$)	0.67	—	0.76	—

DISSOCIATION

It was observed that when the globulins of purified lupin seed meal were examined in different buffers, some variation in the relative proportion of the $s_{7.8}$ and $s_{11.6}$ components occurred. Thus at $I = 0.31$, pH = 7.0, the sedimentation diagrams (Fig. 4(a)) contain a larger proportion of the fast $s_{11.6}$ component, while at $I = 0.2$, pH = 8.8 (Fig. 4(b)) the position is reversed and the sedimentation diagrams contain a larger proportion of the slow $s_{7.8}$ component. At $I = 0.1$, pH = 8.8 (Fig. 4(c)) a major $s_{7.4}$ component was obtained and only a small concentration of the $s_{11.6}$ component. The main peak in this case was rather asymmetric and showed a faster advancing edge.

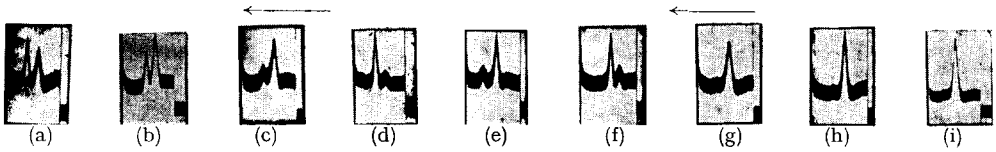

Fig. 4. Sedimentation diagrams of lupin seed proteins.

Electrophoresis carried out in the same buffer (Fig. 1(d)) showed a large concentration of an electrophoretic fast component, which, as shown in Table I, is associated with the $s_{11.6}$ component. It would therefore appear that although the $s_{11.6}$ component is converted into a component similar to the $s_{7.8}$ component with respect to sedimentation velocity, this is not true of electrophoretic properties.

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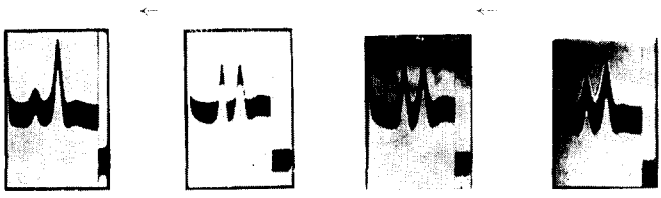
A similar examination was carried out on fractions A and B. Fig. 4(d) showing a major $s_{11.6}$ component gives the sedimentation diagram of fraction A in buffer $I = 0.31$, pH = 7.0. This solution was dialysed overnight against buffer $I = 0.1$, pH = 8.8 (Fig. 4(e)). The $s_{11.6}$ component is hereby almost quantitatively converted into a component of sedimentation constant of 7.1 S.U. Next, this solution was dialysed back to $I = 0.31$, pH = 7.0 and, as shown in Fig. 4(f), the $s_{11.6}$ component was formed again and the obtained sedimentation diagrams were exactly similar to the original ones (compare Fig. 4(d) and 4(f)). Hence, fraction A forms a reversible dissociation-association system. It is noticed that the sedimentation constant of dissociated fraction A (7.1 S.U.) is somewhat lower than that of fraction B (7.8 S.U.). The asymmetric peak, composite of dissociated $s_{11.6}$ component and $s_{7.8}$ component observed in Fig. 4(c), probably arises from the small difference in sedimentation constants of the two components.

Fig. 4(g), 4(h) and 4(i) contain respectively sedimentation diagrams of fraction B in buffers of $I = 0.3$, pH = 7.0; $I = 0.5$, pH = 7.0 and $I = 0.1$, pH = 8.8. No change was observed in the sedimentation diagrams and it would thus appear that fraction B is stable with respect to these changes, and therefore is not the same as the dissociated component, $s_{7.1}$.



(a) (b) (c) (d)

Fig. 5. Sedimentation diagrams of lupin seed proteins.

The influence of ionic strength and pH on the dissociation–association equilibrium of fraction A was now examined more closely. A solution of fraction A in buffer $I = 0.1$, pH = 8.8 (Fig. 5(a)) containing mainly the $s_{7.1}$ component was dialysed for 24 hours against buffer $I = 0.5$, pH = 8.8 and the sedimentation diagrams of Fig. 5(b) were obtained. Further storage (52 hours Fig. 5(c)) at room temperature showed no apparent change and hence the equilibrium was probably attained in 24 hours. As shown, these sedimentation diagrams now contain about equal amounts of both the $s_{7.1}$ and $s_{11.6}$ components. In a similar way Fig. 5(d) gives sedimentation diagrams of fraction A at $I = 0.2$, pH = 8.8. By comparing the different diagrams of Fig. 5 the decrease in relative concentration of the $s_{11.6}$ component with decreasing ionic strength is clear.

Dissociation of fraction A was found to occur at high pH values and low salt concentrations. Reversal of such dissociation was observed at high pH values with increasing salt concentration, but complete reversal was rapidly accomplished by increasing the salt and lowering the pH at the same time.

MOLECULAR WEIGHTS

Weight average values of the diffusion coefficients were obtained as the slope of plots (Fig. 6) of the second moment (σ^2) of the schlieren curves against $2t$ (t being time after boundary formation). Thus the experimental mean values of $3.16 \cdot 10^{-7}$, $3.86 \cdot 10^{-7}$

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and $4.20 \cdot 10^{-7}$ cm^2 sec^{-1} were respectively obtained for undissociated fraction A, dissociated fraction A and formation B. Using these diffusion coefficients and the sedimentation constants of the components, the molecular weights and frictional ratios of Table II were found.

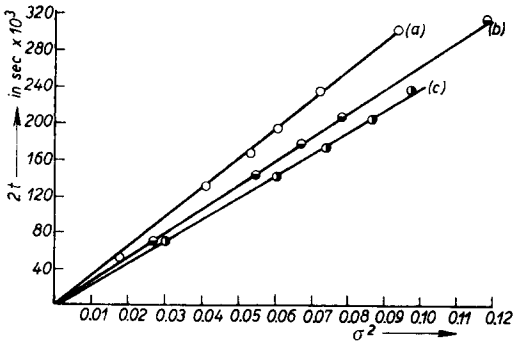

Fig. 6. Plot of second moment (σ^2) against $2t$ for diffusion of (a) undissociated fraction A, (b) dissociated fraction A and (c) fraction B.

TABLE II

SEDIMENTATION MOLECULAR WEIGHTS OF FRACTIONS A AND B

	Fraction A	*Dissociated Fraction A*	*Fraction B*
D^0_{20} cm^2 sec^{-1}	3.16×10^{-7}	3.86×10^{-7}	4.20×10^{-7}
S^0_{20} Svedberg units	11.6 ± 0.2	7.15 ± 0.10	7.79 ± 0.23
Partial specific volume	0.735[3]	0.735[3]	0.752[3]
Molecular weight	336,000	170,000	181,000
Frictional ratio	1.46	1.50	1.34

The properties of the two globulins of lupin seed are very similar to that of vicilin and legumin from seeds of peas reported by DANIELSSON[3]. Not only are the molecular weights very similar, but the method of fractionation applied to the separation of the pea globulins gave almost as good results when used for the fractionation of lupin seed globulins.

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SUMMARY

A physico-chemical study has been made of the globulins from blue lupin seed (*Lupinus angustifolius*). By comparing electrophoretic and ultracentrifugal results, it was found that yellow colouring substances occurring in lupin seed interact with the proteins. These have been removed by ethanol-water and water extraction. Purified lupin seed contains two different globulins, which have been

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separated by fractional precipitation. The molecular weights found for the two globulins are 336,000 and 181,000. The high molecular weight globulin can be reversibly dissociated into a component of 170,000 molecular weight, dissociation being favoured by low ionic strength and high pH. The globulin with molecular weight 181,000 is not identical with dissociated globulin of weight 170,000.

RÉSUMÉ

Les auteurs ont procédé à une étude physico-chimique de la globuline de la graine de lupin bleu (*Lupinus angustifolius*). En comparant les résultats de l'électrophorèse et de l'ultracentrifugation, il apparaît que des substances colorantes jaunes de la graine réagissent avec les protéines. Ces substances peuvent être éliminées par extraction à l'éthanol aqueux et à l'eau. La graine de lupin purifiée contient deux globulines différentes, qui ont été séparées par précipitation fractionnée. Leurs poids moléculaires sont 336,000 et 181,000. La globuline de poids moléculaire élevé peut être dissociée réversiblement en un constituant de poids moléculaire 170,000, la dissociation étant favorisée par une force ionique faible et un pH élevé. La globuline de poids moléculaire 181,000 n'est pas identique à la globuline dissociée de poids moléculaire 170,000.

ZUSAMMENFASSUNG

Es wurde das Globulin aus Samen der blauen Lupine (*Lupinus angustifolius*) physiko-chemisch untersucht. Durch den Vergleich der elektrophoretischen und ultrazentrifugalen Resultate wurde festgestellt, dass die gelbfärbenden Substanzen, die im Lupinensamen enthalten sind, auf die Proteine einwirken. Diese Substanzen wurden durch Ätheylalkohol-Wasser- und Wasser-Extraktion entfernt. Der so gereinigte Lupinensamen enthält zwei verschiedene Globuline, welche durch fraktionierte Präcipitation getrennt wurden. Das Molekulargewicht der beiden Globuline wurde zu 336,000 und 181,000 gefunden. Das höher molekulare Globulin kann reversibel dissoziieren in eine Komponente mit einem Molekulargewicht von 170,000. Die Dissoziation wird begünstigt durch niedrige Ionenstärke und hohen pH. Das Globulin mit dem Molekulargewicht von 181,000 ist nicht identisch mit dem dissoziierten Globulin von Molekulargewicht 170,000.

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