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Fractionation by Gel Exclusion HPLC of Proteins from Acidic and Alkaline Extractions of *Phaseolus* Beans

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ABSTRACT

The proteins extracted from Phaseolus vulgaris *(white kidney, navy) beans and* P. lunatus *(baby lima, large lima) beans by sodium hydroxide (NaOH) solution and citric acid (CA) solutions were fractionated by gel exclusion high-pressure liquid chromatography. The isoelectric amorphous proteins from the NaOH extraction and bipyramidal crystalline and spheroidal proteins from the CA extractions were also fractionated. Both the NaOH and the CA extracts of the beans contained 8–10 fractions. The proteins isolated from the extracts of the* P. vulgaris *beans were comprised predominantly of a relatively large molecular weight fraction, regardless of the microstructure of the protein precipitate; several of the fractions which were relatively major components of the extracts were not found in the precipitates obtained from the extracts. The differences in behaviour between the extracts and protein isolates of the different beans might be related to genetic variations.*

INTRODUCTION

The preparation of proteins from dried bean seeds and investigations on the properties of the proteins have been the subject of numerous reports. A methionine-rich protein isolate has been prepared recently from *Phaseolus vulgaris* beans (Apostolatos, 1984*a*). Micellar proteins from legumes have been prepared (Murray *et al.*, 1978) and investigations on their thermodynamic properties have been reported (Arntfield & Murray, 1981;

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Arntfield *et al.*, 1985). Proteins of different crystalline microstructure were prepared by Bourdillon (1951); the phytate and carbohydrate content of the different microstructures were determined. Several studies have been reported on the preparation and properties of specific protein fractions from legume seeds; these include a glycoprotein fraction, isolectins and isoelectric fractions from *P. vulgaris* (Pusztai & Watt, 1970; 1974) and trypsin inhibitors from *P. vulgaris* (Apostolatos, 1984*b*). The general properties of dry bean proteins have been reviewed by Chang & Satterlee (1982).

The fractionation of bean proteins by electrophoresis, chromatography and ultracentrifugation is often carried out to determine the constitution of the proteins. Recent work on the fractionation of lima bean proteins (Ologhobo, 1984), *P. vulgaris* bean proteins (Yanagi *et al.*, 1984) and three legume seed proteins (Yanagi, 1983) have been reported. Previous studies (Alli & Baker, 1980) in our laboratory have shown that proteins prepared from *Phaseolus* beans by extraction of the ground beans with organic acid solutions (citric acid, malic acid) followed by refrigeration (to precipitate the proteins) exhibited crystalline microstructures; this study also demonstrated that proteins prepared from the same beans by extraction of the ground beans with alkaline solution followed by adjustment of the pH of the extract (to precipitate the proteins), exhibited amorphous microstructure. The contents of phytate and carbohydrates, as well as the electrophoretic behaviour (basic and acidic conditions, in presence or absence of dissociating agents) of the proteins with the different microstructures were also reported (Alli & Baker, 1981; 1983). It has been suggested (Melynchyn, 1969) that the crystalline bean proteins contain less occluded impurities and are therefore in a more refined state than the proteins prepared by isoelectric precipitation. In the present study, fractionation by gel exclusion high pressure liquid chromatography was conducted to determine the nature of the proteins which constitute the crystalline and non-crystalline microstructures which have been described previously.

MATERIALS AND METHODS

Materials

Commercial samples of dried white kidney beans and navy beans (*P. vulgaris*) and baby lima beans and large lima beans (*P. lunatus*) were purchased from a local store. The beans were ground (Mikro-sample mill, Pulverizing Machinery Ltd, Summit N.J.) to pass through a 1 mm sieve and stored in Mason jars.

Extraction of beans and protein precipitation

Extraction with alkaline solution

Samples (10 g) of ground beans were extracted with NaOH solution (0·02%, 100 ml) using the procedure of Fan & Sosulski (1974). A portion of the extract was filtered (Millex-HV filter, Millipore Corporation, Bedford, MA), diluted with phosphate buffer (pH 6·8) and a sample (20 μl) was subjected to gel exclusion HPLC. The remainder of the extract was adjusted to pH 4·5 by dropwise addition of HCl (2N). The material which precipitated (isoelectric protein) was recovered by centrifugation. A sample of the precipitate was dissolved in phosphate buffer, the mixture was filtered (Millex-HV filter), and then subjected to HPLC. The supernatant which remained after the isoelectric protein was recovered was diluted with phosphate buffer and filtered; a sample (20 μl) of the filtrate was subjected to HPLC.

Extraction with citric acid solution

The ground beans were extracted with citric acid solutions in order to prepare crystalline proteins using the procedure of Melynchyn (1969). The beans were extracted using conditions (concentration of acid, pH of extractant) which were determined previously (Alli, 1977) to produce bipyramidal crystalline microstructures and spheroidal microstructures. The conditions of extraction which were employed for the four beans are shown in Table 1. The proteins were precipitated from these extracts by refrigeration (5°C, 18 h) and samples of the extracts, the protein precipitates and the supernatants were subjected to HPLC.

TABLE 1

Concentration and pH of Citric Acid Solution used to Prepare Bipyramidal and Amorphous Microstructures from Four Types of *Phaseolus* Beans

Bean	*Concentration, pH of citric acid solution*	*Microstructure of precipitate*
White kidney	0·08N, pH 5·5	Bipyramidal crystalline
	0·60N, pH 3·0	Spheroidal
Navy	0·20N, pH 5·0	Bipyramidal crystalline
	0·60N, pH 3·5	Spheroidal
Baby lima	0·10N, pH 3·0	Bipyramidal crystalline
	0·20N, pH 4·5	Spheroidal
Large lima	0·10N, pH 3·0	Bipyramidal crystalline
	0·20N, pH 4·5	Spheroidal

Gel exclusion HPLC analysis

Chromatographic analysis was performed using a dual pump system (model 2150 HPLC—Produkter AB, Bromma, Sweden). The proteins were separated on a gel exclusion column (TSK G3000SW, LKB ultropac gel, 0·75 × 60 cm) and detected (206 nm) using a spectral UV detector (LKB model 2140). The chromatographic data were collected by means of an IBM XT personal computer. Integration of the peaks and presentation of the chromatograms were carried out with the aid of a Nelson 2600 chromatography software (Nelson Analytical Inc., Cupertino, CA). Conditions of chromatography were as follows: elution buffer–phosphate buffer (0·1M, pH 6·8) containing sodium chloride; buffer flow rate—0·5 ml min^{-1}.

Estimation of average molecular weight of proteins

The gel exclusion chromatographic procedure was calibrated (for estimating average molecular weight of the protein fractions) using a set of seven protein standards (cytochrome c, MW = 12 500; chymotrypsinogen A, MW = 25 000; egg albumin, MW = 45 000; bovine serum albumin, MW = 68 000; aldolase, MW = 158 000; beef liver catalase, MW = 240 000; ferretin, MW = 450 000). The average molecular weights of the proteins in the samples which were chromatographed were estimated using the equation of Andrews (1964).

RESULTS AND DISCUSSION

White kidney beans and navy beans (*P. vulgaris*)

Figure 1A shows the chromatograms obtained from the sodium hydroxide extract, isoelectric protein and the supernatant recovered after isolation of the isoelectric protein from white kidney bean. At least 10 fractions were separated from the extract (chromatogram i). The proportion (of the total protein fractionated) represented by each fraction, along with the average molecular weights and the protein fractions as determined by gel exclusion HPLC, is shown in Table 2. Fraction 2 (average MW = 230 000) represented approximately 58% of the total proteins fractionated. Fraction 3 (average MW = 100 000) represented 18% of the total protein fractionated. The isoelectric precipitate (chromatogram iii, Fig. 1A) comprised principally Fraction 2 (average MW = 230 000); this fraction represented 82% of the total proteins present in the precipitate (Table 2). Chromatogram ii (Fig. 1A) shows the fractions present in the supernatant recovered after the precipitation of the isoelectric protein. Fraction 2

TABLE 2

Proportion of Each Fraction (As Percent of Total Protein Fractionated) in Extracts, Precipitates, and Supernatants obtained from White Kidney Beans

Fraction No.[a]	*Average MW of fractions*	*Percentage represented by each fraction*								
		NaOH extraction			*Citric acid extraction*					
					0·8N pH 5·5			*0·6N pH 3·5*		
		Ext[b]	*Ppt*[c]	*Supt*[d]	*Ext*	*Ppt*[e]	*Supt*	*Ext*	*Ppt*[f]	*Supt*
1	550 000	3	5	0	2	4·5	1	1	2	0
2	230 000	58	82	1	31	95	9	16	92	4
3	100 000	18	13	35	22	tr[g]	35	18	tr	20
4	32 000	7	0	19	10	0	15	5	0	7
5	21 000	10[h]	0	33	19	0·5	22	48	6	58
6	18 000	.	tr	.	3	0	7	8	0	5
7	15 000	.	0	.	.	0	.	.	0	.
8	13 500	1	0	3	6	0	4	.	0	.
9	10 000	1	0	2	3	0	1	.	0	.
10	6 000	2	0	7	4	0	6	4	0	6

[a] From Figs 1A and 1B.
[b] Extract.
[c] Isoelectric precipitate (amorphous).
[d] Supernatant.
[e] Bipyramidal crystalline microstructures.
[f] Spheroidal microstructures.
[g] Present in trace amounts.
[h] Co-eluted peaks.

(average MW = 230 000), which was the principal fraction in the extract and the precipitate, was virtually absent from the supernatant. This suggests that the isoelectric precipitation of the protein from the sodium hydroxide extract resulted in almost complete removal of the principal fraction (Fraction 2; average MW = 230 000) from the extract.

Figure 1B shows the chromatogram (i) obtained from the citric acid extract (0·08N citric acid, pH 5·5) of white kidney bean. The fractionation of this extract was similar to that of the sodium hydroxide extract. Fraction 2 (average MW = 230 000) was the principal fraction and represented 31% of the total protein fractionated. The citric acid extract contained relatively higher proportions of the low MW fractions than the sodium hydroxide extract. Chromatogram iii (Fig. 2B) shows that the bipyramidal crystalline protein obtained from the citric acid extract (0·08N, pH 5·5, citric acid) comprised principally Fraction 2 (average MW = 230 000); this fraction represented 95% of the total protein fractionated (Table 2). This fraction

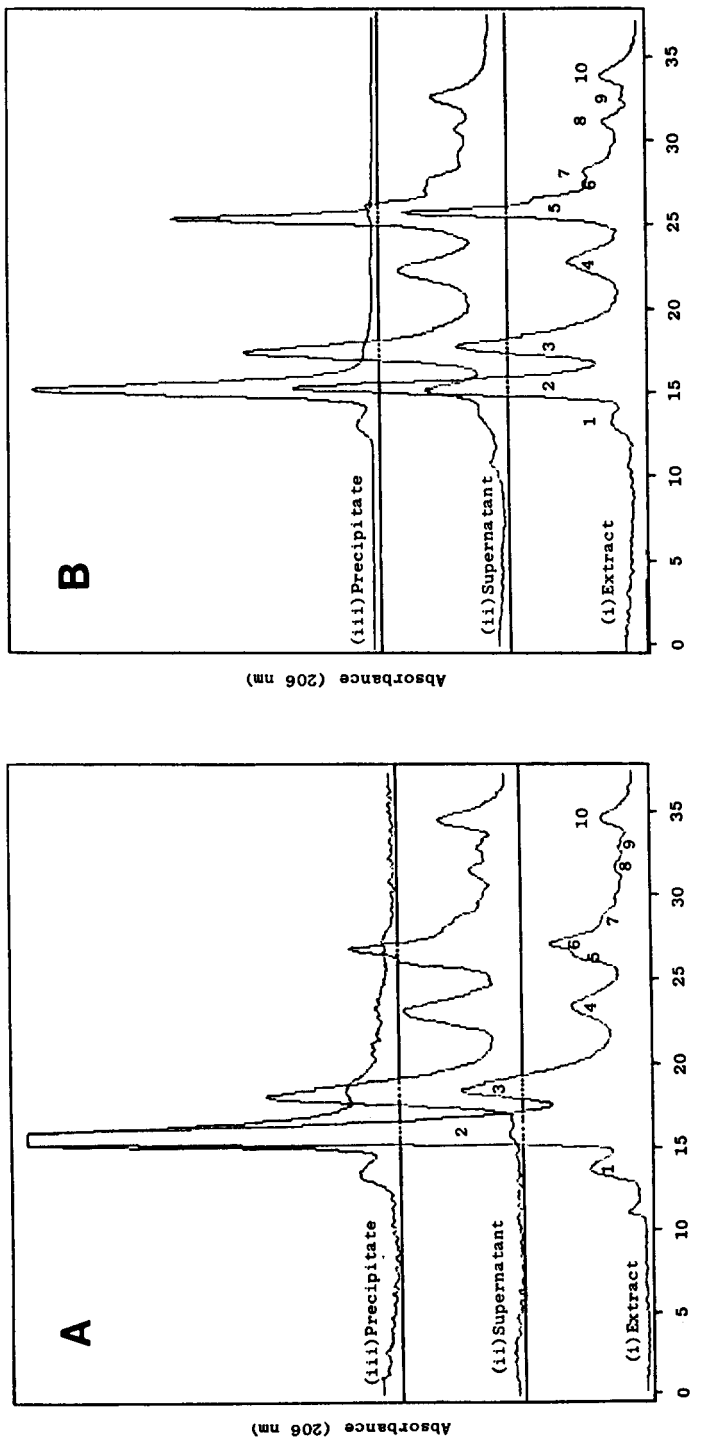

Fig. 1. Gel-exclusion HPLC chromatograms of extracts (i), supernatants (ii) and protein precipitates (iii) obtained from *Phaseolus vulgaris* beans. A. Chromatograms from NaOH extraction of white kidney bean. B. Chromatograms from citric acid (0·08N, pH 5·5) extraction of white kidney bean.

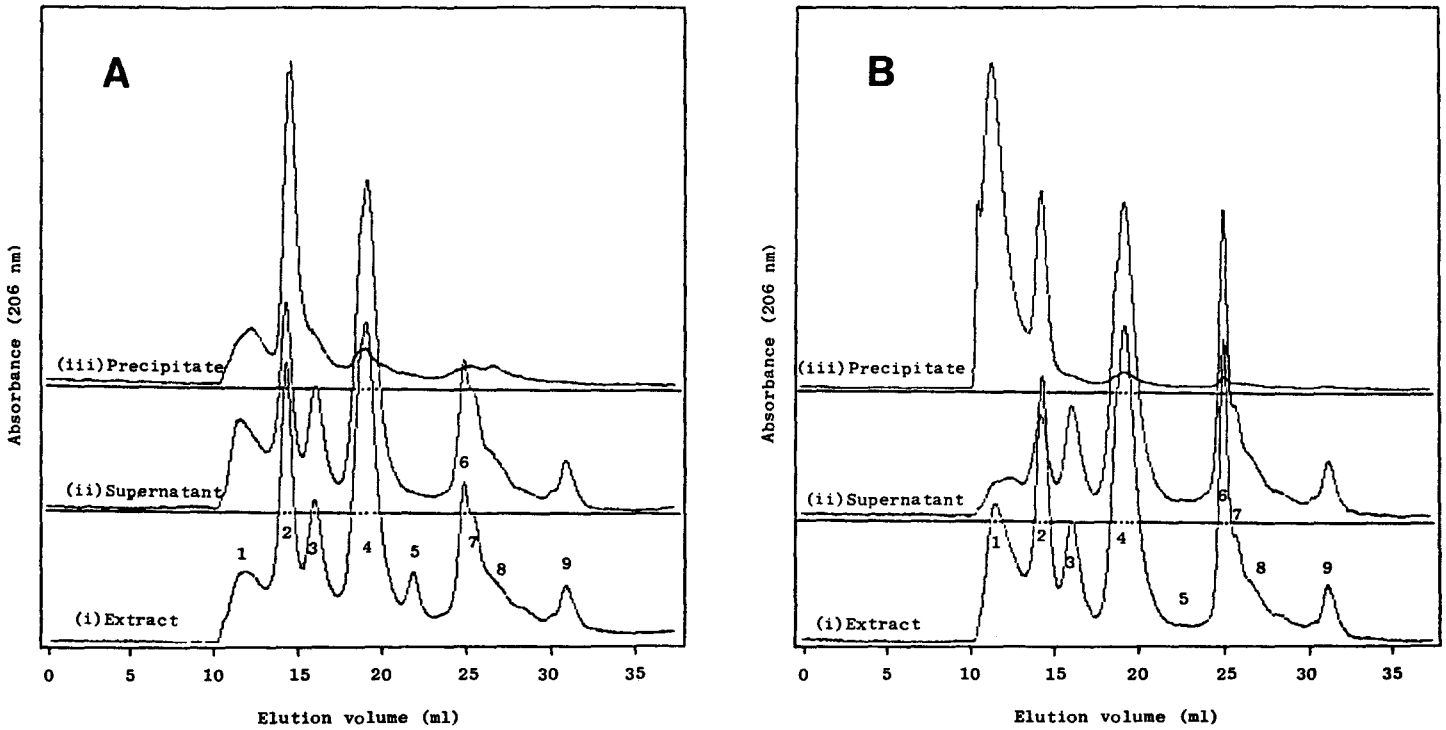

Fig. 2. Gel-exclusion HPLC chromatograms of extracts (i), supernatants (ii) and protein precipitates (iii) obtained from *Phaseolus lunatus* beans. A. Chromatograms from NaOH extraction of baby lima bean. B. Chromatograms from citric acid (0·1N, pH 3·0) extraction of baby lima bean.

was also the principal component of the isoelectric (amorphous) protein. Fractionation of the supernatant recovered after removal of the proteins from the citric acid extract (chromatogram ii, Fig. 1B) indicates that Fraction 2 (average MW = 230 000), which was the principal component in the citric acid extract, represented only 9% of the total protein fractionated from the supernatant. This supports the suggestion that precipitation of the bipyramidal crystalline protein, as well as the isoelectric (amorphous) protein, is associated with the removal of a large proportion of Fraction 2 from the citric acid extract and the sodium hydroxide extract.

The result of the fractionation of the citric acid extract (0·6N, pH 3·0, citric acid) of the white kidney bean is shown in Table 2. The number of fractions (10) was similar to that obtained from the citric acid (0·08N, pH 5·5) extract. However, the lower pH extract contained a relatively high proportion of fraction 5 (average MW = 21 000); this fraction represented 48% of the total protein fractionated from this extract (pH 3·0, 0·6N) but only 18% of the total protein fractionated from the 0·08N citric acid extract (pH 5·5) and less than 10% from the sodium hydroxide extract. The spheroidal protein prepared from the 0·6N citric acid extract (pH 3·0) behaved in a similar manner as the bipyramidal crystalline protein of the white kidney beans. These results suggest that there is little relationship between the microscopic structure of the precipitated protein and the constitution of the proteins when fractionated by gel-exclusion HPLC.

Fractionation of the sodium hydroxide extract, the isoelectric precipitate and the supernatant recovered after removal of the isoelectric precipitate from the sodium hydroxide extract, of the navy bean, were similar to those of the white kidney bean; these two beans are cultivars of *P. vulgaris*.

The average MW (200 000) of Fraction 2 in the extracts, precipitates and supernatants obtained from the navy bean were found to be somewhat lower than the average MW (230 000) of the Fraction 2 in the white kidney beans. In addition, the isoelectric precipitate of the navy bean contained a fraction of relatively low MW (approx. 18 000) which was present in trace amounts only in the isoelectric precipitate of the white kidney bean. These differences could be related to the genetic variation between the two varieties of *P. vulgaris* beans.

Baby lima beans and large lima beans (*P. lunatus*)

Figure 2A shows the chromatogram (i) of the sodium hydroxide extract of the baby lima bean. Table 3 shows the proportion (as a percentage of the total protein fractionated) represented by each fraction. The principal fraction in this extract was Fraction 4 (average MW = 50 000) which

TABLE 3

Proportion of Each Fraction (As Percent of Total Protein Fractionated) in Extracts, Precipitates, and Supernatants obtained from Baby Lima Beans

		Percentage represented by each fraction								
		NaOH extraction			*Citric acid extraction*					
					0·1 N pH 3·0			*0·2 N pH 4·5*		
Fraction No.[a]	*Average MW of fractions*	*Ext*[b]	*Ppt*[c]	*Supt*[d]	*Ext*	*Ppt*[e]	*Supt*	*Ext*	*Ppt*[f]	*Supt*
1	700 000	9	18	12	16	70	6	12	9	11
2	270 000	19	58[g]	16	18	26	9	16	20	15
3	129 000	12	.	11	9	tr[h]	11	10	9	10
4	50 000	32	15	26	32	3	40	31	28	32
5	24 000	4	tr	tr	tr	tr	0	tr	tr	tr
6	11 000	21	9	22	22	1	30	28	29	29
7	9 000	.	.	.	.	0	.	.	.	.
8	7 000	.	.	.	.	0	.	.	.	.
9	4 000	3	0	3	3	0	4	3	3	3

[a] From Figs 2A and 2B.
[b] Extract.
[c] Isoelectric precipitate (amorphous).
[d] Supernatant.
[e] Bipyramidal crystalline microstructures.
[f] Spheroidal microstructures.
[g] Co-eluted peaks.
[h] Present in trace amounts.

represented 32% of the total protein fractionated; this fraction was not found in the extracts of the *P. vulgaris* beans. Fraction 2 (average MW = 270 000) represented 19% of the total protein fractionated. This is somewhat different from the *P. vulgaris* beans in which the fractions of relatively high MW (average MW = 200 000–230 000) were the principal fractions of the extracts. Chromatogram iii (Fig. 2A) shows that Fraction 2 (average MW = 270 000) was the principal fraction (58% of total protein fractionated, Table 3) in the isoelectric precipitate. Fraction 4 constituted only 15% of the proteins in the isoelectric precipitate. The results (chromatogram ii, Fig. 2A) suggest that there was a substantial decrease in the relative amount of Fraction 2 (average MW = 270 000) from the extract as a result of the precipitation of the protein from the sodium hydroxide extract.

The protein constitution of the citric acid extract (0·1N, pH 3·0) was similar to that of the sodium hydroxide extract (Figs 2A and 2B,

chromatogram i). Chromatogram iii (Fig. 2B) shows the fractions present in the bipyramidal crystalline protein isolated from the 0·1N citric acid extract (pH 3·0). The protein precipitate was comprised principally of relatively large MW fractions; Fraction 1, 70% (average MW = 700 000) and Fraction 2, 26% (average MW = 270 000), (Table 3). Fraction 1 comprised less than 5% of the total protein of the isolates from the *P. vulgaris* beans and was also a relatively minor fraction (<6% of the total protein fractionated) in the isoelectric protein of the navy beans. Fractionation of the supernatant after separation of the bipyramidal crystalline protein from the citric acid extract indicates a substantial loss of Fraction 1 as well as the loss of Fraction 2; this supports the suggestion that the bipyramidal crystalline protein of the baby lima bean is comprised principally of these two fractions. Fractionation of the spheroidal protein indicates the presence of a relatively high proportion of low MW fractions; Fractions 6, 7 and 8, (average MW = 7000–11 000) represented 29% of the total protein fractionated while Fraction 4 (average MW = 50 000) represented 28% of the total protein fractionated. The results from the fractionation of the large lima bean (a variety of *P. lunatus*) were closely similar to those obtained for the baby lima bean. This may be related to the fact that the large lima bean and baby lima bean belong to the same species.

CONCLUSION

The fractionation of the various protein extracts and isolates suggests the following: (i) proteins showing different microstructures could have similar constitution (e.g. the isoelectric (amorphous) protein and the bipyramidal crystalline protein of white kidney bean) or different constitution (e.g. the isoelectric (amorphous) protein and the bipyramidal crystalline protein of the baby lima bean), (ii) the protein constitution of the extracts from the beans is affected by the pH of the extracting solution (e.g. under acidic conditions of extraction there is the tendency for larger quantities of relatively low MW fractions to be extracted when compared with alkaline conditions of extraction), (iii) the composition of the extracts from which the proteins precipitate does not appear to affect the composition of the proteins which are precipitated and (iv) differences in the chromatographic behaviour of the extracts and protein precipitates of the different beans could be related more to genetic differences than to differences in microstructures of protein precipitates. This suggests that, like gel electrophoresis (Lowry *et al.*, 1974) HPLC fractionation of bean proteins could be useful as a tool for detecting differences in proteins occurring in different species of seeds of the same genus.

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