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Studies on the Extraction of Nitrogenous and Phosphorus-Containing Materials from the Seeds of Kidney Beans (*Phaseolus vulgaris*)

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1. The conditions of extracting nitrogenous, phosphorus-containing and glucosamine-containing components of the seeds of kidney bean have been studied. 2. The dispersing of proteins was incomplete below pH 7, and the exact amount of protein extracted depended on the pH and the ionic strength of the solvent. 3. The extraction of proteins was practically complete in the range pH 7–9, but the relative amounts of the individual proteins obtained still depended on the pH of the extracting media, indicating a pH-dependent association–dissociation reaction between the protein molecules present. 4. The extraction of phosphorus-containing material showed an optimum at pH 6–7, and only a part of this was removed on dialysis. The precipitates obtained with trichloroacetic acid, on the other hand, retained very little phosphorus-containing material. 5. The significance of these findings is discussed.

In the preceding paper (Pusztai, 1965) the occurrence and the isolation of proteins containing D-glucosamine and D-mannose were described. The evidence presented there indicated that these sugar residues are joined to the proteins of the kidney bean by a strong linkage that may well be covalent in nature. The results of the experiments described also pointed to the possibility that a major part of the proteins of the seeds contained sugar residues.

In the present paper the quantitative and certain other aspects of extracting proteins and glycoproteins are described and compared with those for other nitrogenous and phosphorus-containing components of the seed.

MATERIALS AND METHODS

Kidney beans. The kidney bean used for this work was a new and different variety from that used for previous work. It is called 'haricot kidney bean' and sold locally by W. Smith and Sons (Aberdeen).

Kjeldahl nitrogen and amino sugars. These were estimated as described by Pusztai (1965).

Phosphorus. Total phosphorus was determined by the method of Fiske & Subbarow (1925) and Berenblum & Chain (1938) after incinerating the samples with sulphuric acid–nitric acid and 60% (w/v) perchloric acid respectively.

Production of antisera. Rabbit antisera against the protein mixtures of the extracts of kidney beans were prepared as follows. Rabbits were immunized by a course of 12–14 intravenous injections, spread over 6–7 weeks, of seed proteins (5–15 mg. of protein/injection) precipitated with alum and adjusted to pH 7·5. The animals were killed and their blood was collected 5 days after the last injection.

Immunoelectrophoresis. A micro method based on that of Grabar & Williams (1955) and Hirschfeld (1960) was used. The conditions were as follows: 2 ml. of a 1% agar gel in 0·05 M-tris–acetate buffer, pH 8·3, was melted and spread on a glass micro-slide (75 mm. × 25 mm.) with a pipette. Materials were applied in preformed wells 27·5 mm. from the cathode end of the slide. Electrophoresis was carried out with a potential gradient of 5·5 v/cm. across the slide for 2 hr.

Standard extraction procedure. Finely ground kidney-bean meal (300 mg.) was placed in a polypropylene centrifuge tube with the extracting solvent (10 ml.) and thoroughly stirred. The contents of the tube were kept rocking gently overnight in a cold room and then centrifuged. Portions of the clear supernatant were used for estimating nitrogen, phosphorus, hexosamine content and the other tests. Portions were also taken for precipitating proteins in the seed extract with trichloroacetic acid (final concn. 7·5%, w/v). The precipitates were washed thrice with aq. 7·5% trichloroacetic acid solutions and finally dissolved in 0·1 N-NaOH. The nitrogen, phosphorus and hexosamine contents of these solutions were determined.

In a few instances portions of the original extracts were dialysed against buffers of the same pH and composition as that of the dispersing agent used for extracting. The non-diffusible part was made up to a known volume; nitrogen and occasionally phosphorus estimations were performed on these solutions.

High-voltage electrophoresis. This was performed as described by Pusztai (1965).

Chromatography on Sephadex G-200 columns. The experimental conditions were as described by Pusztai (1965). For a semi-quantitative evaluation of the results the

extinction values of the fractions collected were read at 280 and 260 mμ and plotted against the elution volume or fraction number. The relative amounts of proteins of various molecular sizes were calculated in a rough approximation from the area occupied by them on these graphs.

RESULTS

The kidney bean used in the present work ('haricot') was found to give the following analytical data: N (Kjeldahl), 4·50 ± 0·05%; P (total), 0·60 ± 0·02%; glucosamine (after hydrolysis in 0·5 N-hydrochloric acid at 100° for 16 hr.), 0·19 ± 0·01%.

The results of extraction, diffusion and precipitation studies on this kidney bean are summarized in Tables 1 and 2. Table 1 also contains data on the distribution of proteins of various sizes and designated as α, β, γ and δ in order of decreasing molecular size in these extracts as obtained by chromatography on Sephadex G-200 gel columns. A typical example has been given by Pusztai (1965).

The extracts of kidney beans obtained with various dispersing solvents were also subjected to high-voltage electrophoresis in the Hannig apparatus and to immunoelectrophoresis in agar gel. The results of these examinations at the two extreme pH values used in this work, pH 5 and 9, are given in Figs. 1 and 2.

Table 1. *Results of extraction of proteins and other nitrogenous materials from kidney beans*

Experimental details are described in the text. The amount of protein nitrogen extracted is estimated (*a*) from materials precipitated with trichloroacetic acid and (*b*) from the non-diffusible part of the extract.

Dispersing agent	Concn. of dispersing agent (M)	N extracted (g./100 g. of total seed N)	Protein N extracted (g./100 g. of total seed N)		Relative amounts of proteins of various sizes separated by chromatography on Sephadex G-200 columns (% of total protein)			
			(*a*)	(*b*)	α	β	γ	δ
Sodium acetate buffer, pH 5·0	0	27·5	7·4	14·8	—	—	—	—
	0·02	23·8	9·2	—	5	70	15	10
	0·1	35·6	21·6	—	5	70	14	11
	0·2	50·2	36·3	—	—	—	—	—
	0·5	60·6	44·2	—	7	69	14	10
Sodium chloride soln., pH 5·5	0	20·7	9·2	—	—	—	—	—
	0·02	29·7	15·6	22·8	7	67	16	10
	0·1	46·0	29·7	—	10	65	16	9
	0·2	53·4	41·6	—	11	63	16	10
	0·5	52·0	40·7	—	20	56	15	9
Sodium phosphate buffer, pH 6·0	0	38·5	19·3	25·1	—	—	—	—
	0·02	37·2	20·7	25·6	12	62	14	12
	0·1	62·2	47·0	—	—	—	—	—
	0·2	70·0	54·4	—	25	52	13	10
	0·5	64·4	49·2	—	28	48	14	10
Sodium phosphate buffer, pH 7·0	0	86·6	66·6	72·6	32	46	14	8
	0·02	81·5	63·6	—	30	48	13	9
	0·1	85·0	67·3	—	—	—	—	—
	0·2	83·0	63·0	—	36	44	12	8
	0·5	72·6	52·6	—	—	—	—	—
Sodium phosphate buffer, pH 8·0	0	84·2	67·9	72·6	—	—	—	—
	0·02	85·0	68·8	—	35	46	11	8
	0·1	84·2	68·8	—	36	44	11	9
	0·2	71·4	57·3	—	—	—	—	—
	0·5	71·0	57·3	—	38	43	11	8
Sodium borate buffer, pH 9·0	0	85·3	71·0	73·5	42	42	10	6
	0·02	82·6	67·9	72·6	42	40	10	8
	0·1	86·6	71·8	73·5	41	41	11	7
	0·2	87·8	73·5	73·5	—	—	—	—
	0·2 [containing NaCl (0·3 M)]	86·0	74·0	—	39	44	10	7

Table 2. *Recovery of phosphorus- and glucosamine-containing materials from kidney beans*

Experimental details are described in the text.

Dispersing agent	Concn. of dispersing agent (M)	P extracted (g./100 g. of total seed P)	P precipitated with trichloro-acetic acid (g./100 g. of total seed P)	Indiffusible P (g./100 g. of total seed P)	Glucosamine extracted (g./100 g. of beans)	Glucosamine insoluble in trichloroacetic acid (g./100 g. of beans)
Sodium acetate buffer, pH 5·0	0	58·4	0	48·4	0·02	0·02
	0·02	58·4	0	48·4	—	—
	0·1	—	0	—	—	—
	0·2	61·5	0	—	—	—
	0·5	63·2	0	—	0·10	0·10
Sodium chloride soln., pH 5·5	0	60·0	0	48·4	—	—
	0·02	58·4	0	48·4	—	—
	0·1	—	0	—	0·06	0·07
	0·2	63·2	0	50·0	—	—
	0·5	66·6	0	53·4	0·11	0·10
Sodium phosphate buffer, pH 6·0	0	71·7	0	58·4	0·04	0·04
	0·02	—	0	—	—	—
	0·1	—	0	—	—	—
	0·2	—	0	—	—	—
	0·5	—	1·7	—	0·12	0·13
Sodium phosphate buffer, pH 7·0	0	81·8	1·7	58·4	0·18	0·18
	0·02	—	1·7	—	—	—
	0·1	—	1·7	—	—	—
	0·2	—	3·3	—	—	—
	0·5	—	1·7	—	0·16	0·15
Sodium phosphate buffer, pH 8·0	0	65·0	3·3	43·3	0·17	0·17
	0·02	—	3·3	—	—	—
	0·1	—	3·3	—	—	—
	0·2	—	5·0	—	—	—
	0·5	—	5·0	—	—	—
Sodium borate buffer, pH 9·0	0	51·7	3·3	36·7	0·18	0·18
	0·02	50·0	5·0	—	—	—
	0·1	—	6·7	—	0·18	0·18
	0·2	—	6·7	—	—	—
	0·2 [containing NaCl (0·3 M)]	—	5·0	—	0·18	0·18

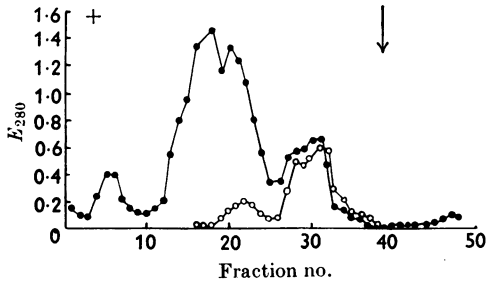

Fig. 1. Separation of the constituents of kidney bean by high-voltage electrophoresis (conditions: Hannig apparatus; 0·1 M-tris–acetate buffer, pH 8·3; 30 v/cm.; 170 mA; rate of buffer flow in the cuvette, 50 ml./hr.; sample introduction rate, 2 ml./hr.; passage time 7 hr.; sample introduction point, as indicated by the arrow). The extinction values of the separated fractions were read at 280 mμ. ○, Materials obtained by extracting with 0·02 M-sodium acetate buffer, pH 5·0; ●, materials obtained by extracting with 0·02 M-borate buffer, pH 9·0.

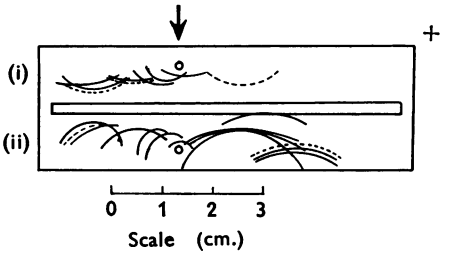

Fig. 2. Separation of the antigens of kidney bean by immunoelectrophoresis (conditions: 1%, w/v, agar gel; 0·05 M-tris–acetate buffer, pH 8·3; 5·5 v/cm. for 2 hr.). (i) pH 5·0 extract (0·02 M-sodium acetate buffer); (ii) pH 9·0 extract (0·02 M-borate buffer). Anti-(pH 9·0 extract) serum was used. Incubation was at 37° for 16 hr.

DISCUSSION

There is now sufficient experimental evidence to support the view that most of the proteins extracted from the seeds of higher plants contain sugar residues, and that these residues, the most important of which are D-glucosamine, probably N-acetylated, and D-mannose, are in a firm combination with the peptide part of the molecule (Pusztai, 1964*a*,*b*, 1965). The conditions of extraction of proteins and that of other constituents of the seeds that might conceivably have some effects on the recovery and a subsequent study of glycoproteins have now been studied.

Pusztai (1965) showed that tris–acetate buffer, pH 8·3, brings into solution all the protein-bound glucosamine of the seed, but no data on the recovery of proteins under the same conditions have been reported. The results of the extraction studies (Table 1) show the importance of the conditions such as pH and ionic strength of the dispersing media on the amount of total N, proteins etc. extracted from the seeds. The recovery of N under various conditions followed a similar pattern to that described for soya beans by Smith & Circle (1938) and Smith, Circle & Brother (1938), with certain exceptions. Below pH 7 the recovery of N is not complete, although by increasing the ionic strength of the dispersing solvents up to a point an increasingly greater proportion of the seed N is solubilized. Above pH 7 the ionic strength has little effect on the dispersibility of the nitrogenous components of the seed, as practically 100% extraction of N can be achieved between pH 7 and 9 at low or moderate ionic strength. Table 1 also contains values for N recovery when unbuffered solutions of sodium chloride, the most common plant-protein dispersing agent, in various concentrations (Osborne, 1924; Smith, Earle, Wolff & Jones, 1959), are used for extracting proteins from kidney bean. The seeds used in the present work give a pH in distilled water of 5·5 and the unbuffered sodium chloride solutions give the same or, with increasing sodium chloride concentration, slightly lower pH values. Under these conditions only a part of the seed N, the exact amount depending on the concentration of the sodium chloride, is extracted. The recovery of the non-protein N is much less affected by changes in the pH and the ionic strength of the dispersing media than that of the non-diffusible N; so the strong pH-dependence of extracting N from the seeds can be attributed to the solubility characteristics of the proteins of kidney beans.

The results of chromatography on Sephadex G-200 columns (Table 1) and, to a certain extent, those of high-voltage electrophoresis (Fig. 1) and immunoelectrophoresis (Fig. 2), show that not only the quantity of the proteins recovered but the quality and the composition of these protein mixtures also depend on the pH and, to a smaller extent, on the ionic strength of the extracting solvent. The proteins of the seed obtained with various extracting agents have been separated by chromatography on Sephadex G-200 columns into four components that may be similar to the four components (two major and two minor) observed in the analytical ultracentrifuge in the extracts of other seeds (Johnson, 1946*a*; Naismith, 1955; Wolf & Briggs, 1956), and, although the resolved proteins still represent more or less complicated mixtures of proteins of similar molecular sizes, the approximate quantities calculated for each group under different conditions of extraction show the effect of the changing conditions on the amount of protein contained in each group. The data in Table 1 indicate that little of the first protein component, α, is extracted at below pH 7 and at low ionic strength and, although the proportion of this component rises with increasing ionic concentration, maximum values cannot be attained. Further, even under conditions when practically all the N is dispersed, i.e. at or above pH 7, the relative amount of this component varies with the pH; e.g. the amount of this component is 32% of the total amount of protein in the pH 7 extract with water, whereas a similar extract at pH 9 contains 42% of the total protein in this form. The overall quantity of protein extracted, however, is practically the same at both pH values. Changes in the relative and the absolute amounts of this protein component with various pH values could be explained by some form of transformation, or perhaps association, of other proteins present, especially as this increase at pH 9 seems to take place at the expense of the second protein component, β, the total amount of proteins remaining the same at both pH values.

Reversible association–dissociation reactions similar to those suggested for several seed proteins (Johnson, 1946*b*; Johnson & Shooter, 1950; Rackis, Smith, Babcock & Sasame, 1957; Briggs & Wolf, 1957; Wolf, Rackis, Smith, Sasame & Babcock, 1958; Wolf & Briggs, 1958; Kretovich, Smirnova & Karyakin, 1961) may play a part in this transformation reaction.

The presence of several protein components in the extracts of kidney beans has also been demonstrated by high-voltage electrophoresis (Fig. 1) and immunoelectrophoresis (Fig. 2). The general patterns obtained in these examinations are similar to those of the chromatography on Sephadex G-200 gel; e.g. little of the proteins migrating to the anode at pH 8·3, the major protein components of the seed, are extracted at below pH 7, indicating that the first component, α, from the Sephadex gel may at least in part be identical with a substantial part

of the negatively charged proteins. No association can be shown by these methods, as molecular size has little influence on the mobility of charged macromolecules in electric field (Johnson & Naismith, 1953; Kondo, Mori & Kajima, 1953). Nevertheless, occasionally some irreversible aggregation of proteins can be shown to have occurred when extracts of the seed had been stored at pH 5 for some time and readjusted to pH 8·3 before subjecting them to immunoelectrophoresis in agar gel. Under these conditions a streaking protein band (which does not diffuse or react with the antibodies present) migrating towards the anode can be observed. It seems likely that this aggregated material can move in the agar gel only under the considerable shearing force brought about by the electric field, and as soon as this force ceases to operate the aggregated protein cannot move or be eluted. Whether this seemingly irreversible aggregation is similar to or a part of the association reaction observed on Sephadex chromatography is not clear, but it is probably responsible for the unsatisfactory resolution obtained on paper electrophoresis of various seed extracts (Bötger, 1958).

Table 2 contains data on the recovery of phosphorus (total, non-diffusible and trichloroacetic acid-precipitated) from the seeds of kidney beans. The presence of phosphorus in these extracts can have an important bearing on the behaviour and extractability of proteins, as according to Fontaine, Pons & Irving (1946) about 90% of the total seed phosphorus is present in the form of phytic acid, which shows a tendency to form complexes with various proteins at acidic pH values. These complexes are sparingly soluble in water or aqueous salt solutions below their isoelectric point, and in this way considerable amounts of protein can be lost from the extracts of the seed. There are methods for the removal of phytic acid based on dialysing the extracts at slightly alkaline pH against buffers or sodium chloride solutions of high ionic strength (McKinney, Sollars & Setzkorn, 1949), or treating the extracts with Dowex 1 resin first and then dialysing them (Smith & Rackis, 1957). These methods were claimed to have removed a large part of phytic acid from the extracts of soya-bean meal.

The present results show an approximate pH optimum for the dispersion of phosphorus-containing material between pH 6 and 7 for kidney beans, a value not far removed from that found by Fontaine *et al.* (1946) for a number of other seeds, with a definite decrease in the amount of phosphorus-containing material solubilized at higher pH values. No complete removal of phosphorus-containing material was found at any of the pH values (pH 5–9) studied by dialysing the various extracts. On the other hand, the protein precipitates obtained with trichloroacetic acid from the same extracts contained little phosphorus. This phosphorus may have come from the small amounts of nucleic acid present in various seeds (Di Carlo, Schultz & Kent, 1955), as phytic acid–protein complexes are completely dissociated at the low pH, and phytic acid, inorganic and low-molecular-weight nucleotide-bound phosphorus are removed in the acidic supernatant solution.

Finally, glucosamine estimations performed on the extracts of the seed of kidney beans and on the protein precipitates obtained from them with trichloroacetic acid show conclusively what has been indicated by Pusztai (1965), namely that the amount of glucosamine extracted depends on the efficiency of the protein extraction, and that the glucosamine once extracted under any conditions described will stay with the proteins when precipitated with acid.

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