The Isolation of Two Proteins, Glycoprotein I and a Trypsin Inhibitor, from the Seeds of Kidney Bean (*Phaseolus vulgaris*)

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1. The isolation of two proteins from the seeds of kidney bean is described. 2. The individual steps in the purification procedure included: extraction of the seeds at pH 9·0, dialysis, first against pH 9·0 and then against pH 5·0 buffers, high-voltage electrophoresis of the proteins soluble at pH 5·0 and chromatography on Sephadex G-200, Sephadex G-75 and DEAE-Sephadex columns. 3. Of the two proteins isolated, the first and larger component was a glycoprotein and its carbohydrate part was mainly composed of D-mannose and D-glucosamine together with smaller amounts of arabinose, xylose and fucose. 4. The second protein component isolated was a trypsin inhibitor and was almost entirely devoid of sugars but contained a firmly bound pinkish-blue pigment. 5. The amino acid composition of the two proteins was determined. 6. The glycoprotein contained very little if any cyst(e)ine but was relatively rich in aromatic amino acids, whereas the trypsin inhibitor had an unusually high cystine content (nearly 15%) but was relatively poor in valine and in aromatic amino acids.

Pusztai (1964*a,b*) suggested that proteins containing amino and neutral sugars firmly bound to the peptide moiety are present in kidney beans. The main sugar constituents of these proteins were isolated, crystallized and identified as D-glucosamine and D-mannose (Pusztai, 1964*a*, 1965*a*). The evidence obtained indicated a firm combination between the amino acid and the sugar residues as all non-hydrolytic techniques used, including extraction with phenol-containing solvents, failed to separate the protein and the carbohydrate moieties (Pusztai, 1965*a*, 1966). At present the chemical nature of the linkage or linkages involved is not known.

The isolation of a glycoprotein from the seeds of kidney bean has recently been described in a short communication (Pusztai, 1965*c*). In the present paper a full account of this work, together with the description of the isolation of a second protein, a trypsin inhibitor, is given. The latter protein, in contrast with the glycoprotein, is virtually free from sugars.

MATERIALS AND METHODS

Kidney bean. This was a variety called 'haricot' and sold locally by W. Smith and Sons (Aberdeen). The results of the chemical analyses for this variety have already been published (Pusztai, 1965*b*).

Nitrogen (*Kjeldahl*) *and amino sugars.* These were estimated as described before (Pusztai, 1964*a*, 1965*a*).

Amide nitrogen. The proteins were digested with 10N-HCl at 37° for up to 3 weeks (Rees, 1946). Portions of these digestion mixtures were taken at various time-intervals, evaporated *in vacuo* at 37° and the residues taken up in a small amount of water. The ammonia liberated from glucosamine and amido groups was distilled by using saturated $Na_2B_4O_7$ in saturated Na_3PO_4 (Tracey, 1952). Similar samples were used for estimating the amount of glucosamine liberated by the acid, and the amide nitrogen values were corrected for the nitrogen content of this amino sugar (Johansen, Marshall & Neuberger, 1960).

Neutral sugars. These were estimated by the phenol–H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers & Smith (1956) and the results are expressed as D-mannose. Estimations of neutral sugars were also carried out by the carbazole–H_2SO_4 method of Seibert & Atno (1946). Extinctions were read at 440 and 534 mμ. The ratio of these is larger than 1·0 for mannose and is less than 1·0 for a number of other sugars. For a few experiments the orcinol–H_2SO_4 method (Winzler, 1955) was also used. The method was standardized for mannose and readings were made at 512 mμ. Quantitative estimations of the individual sugars liberated by hydrolysis with 0·5N-HCl at 100° for 16hr. or with 2N-HCl at 100° for 3hr. were also attempted by the chromatographic method of Wilson (1959). The hydrolysates, which usually contained 2–5mg. of protein/ml., were evaporated *in vacuo*, and the residues were taken up in 2ml. of water and applied to a column (4cm. × 1cm.) of Dowex 50 (X4; H^+ form) resin. The neutral sugars were eluted by washing the column with water (50ml.). The eluates were neutralized by the addition of appropriate amounts of Dowex 2 (CO_3^{2-} form) resin, filtered and the resin was washed with water (25ml.). The filtrate and the washings were combined and evaporated *in vacuo*. The

residues were taken up in known amounts of water and applied to a Whatman no. 1 paper. The individual sugars were separated on paper with, as a solvent mixture, the organic phase from ethyl acetate–pyridine–acetic acid–water (20:8:1:8, by vol.). To check the efficiency of the method, samples of crystalline ovalbumin and bovine serum albumin, to which known amounts of sugars were added, were also subjected to the same procedure. The results of these estimations indicated a mannose content of 1·86% for ovalbumin. This value compares well with the accepted value of about 2% as given by Johansen *et al.* (1960). The recoveries of sugars hydrolysed in the presence of serum albumin were as follows: mannose, 95%; fucose, 94%; arabinose, 97%; galactose, 102%; xylose, 95%.

Methylpentoses. These were estimated by the cysteine–H_2SO_4 method of Dische & Shettles (1948). The results are expressed as L-fucose.

Pentoses. These were determined by the basic cysteine–H_2SO_4 test of Dische (1949). The results are expressed as D-arabinose.

Uronic acids. These were determined by the carbazole–H_2SO_4 technique of Dische (1947).

Amino acid analysis. The proteins were hydrolysed with 5·7N-HCl (glass-distilled) in sealed tubes under nitrogen at 110° for 24, 48 and 72hr. and subjected to amino acid analysis by a method of Moore, Spackman & Stein (1958). Cyst(e)ine and methionine were also estimated from the hydrolysates of performic acid-oxidized proteins by the method of Moore (1963).

Tyrosine and tryptophan estimations. These were made by the spectrophotometric method of Bencze & Schmid (1957). The measurements were made on the protein samples with or without added tryptophan. The similar technique of Goodwin & Morton (1946), when applied to these proteins, gave inconsistent results and it was therefore abandoned.

Chromatography on Sephadex columns and high-voltage electrophoresis. These were carried out as described before (Pusztai, 1965*a*,*b*).

Chromatography on DEAE-Sephadex columns. DEAE-Sephadex A-50 was washed with 2N-NaOH, water, 2N-HCl and water, in that order. The ion-exchanger was finally equilibrated with 0·05M-tris–acetic acid buffer, pH8·3, containing NaCl (0·05M). A column (34cm. × 4cm.) was poured and the protein samples were applied to the top of the ion-exchanger. The chromatogram was developed with a near-linear gradient of NaCl in 0·05M-tris–acetic acid buffer, pH8·3. The NaCl concentration in the effluent was checked by titration with standard 0·1M-$AgNO_3$ solution with a drop of K_2CrO_4 solution as indicator. The effluents were collected by the means of a fraction collector and the contents of the tubes were read at 280mμ with a Beckman DB spectrophotometer to locate the proteins.

EXPERIMENTAL AND RESULTS

The fractionation techniques used in the present work were similar to those described before (Pusztai, 1965c) but two additional steps were incorporated. A typical experiment is now described in detail. Finely ground seeds of kidney bean (130g.) were stirred at 4° for 1hr. with 0·02M-boric acid solution adjusted to pH9·0 with 0·1N-sodium hydroxide solution (500ml.) and then centrifuged. The sediment was re-extracted with buffer (500ml.) and centrifuged. The supernatant contained 4·92g. of N, corresponding to 84·2% of the total seed N. The combined supernatants (870ml.) were dialysed against six changes, each of 10l., of 0·02M-borate, pH9·0, for 72hr. The indiffusible part (1070ml.) contained a total of 4·20g. of N, corresponding to 85·4% of total N extracted. This solution was then dialysed against six changes, each of 10l., of 0·033M-sodium acetate–acetic acid buffer, pH5·0, for 5 days. The resulting heavy precipitate was centrifuged. This fraction contained 3·43g. of N, or 69·7% of total seed N extracted, and was not used in the present study. The clear supernatant (1l.) contained 0·77g. of N, or 15·8% of total extracted N, and was saturated with solid ammonium sulphate (760g./l.). The resulting precipitate was collected by centrifuging, dissolved in and dialysed against 0·1M-tris–acetic acid buffer, pH8·3. The ammonia-free protein solution (about 250ml.) was subjected to electrophoresis in the Hannig (1961) apparatus under conditions similar to those previously described (Pusztai, 1965c). The extinction values of the contents of the 48 collection tubes (numbered from the anode side) were read at 280mμ to locate the position of the proteins (Fig. 1). The contents of tubes 20–23 were

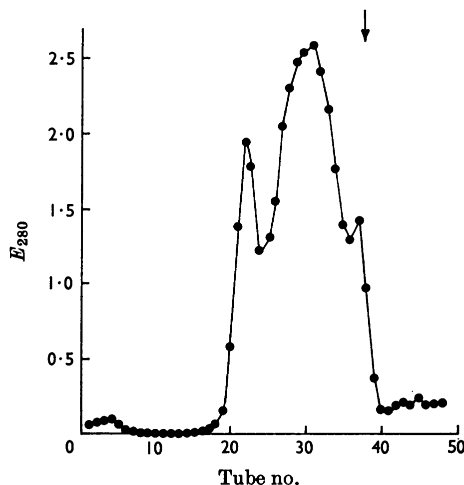

Fig. 1. Separation of the proteins of kidney bean soluble in 0·033M-sodium acetate buffer, pH5·0, by high-voltage electrophoresis (conditions: Hannig apparatus; 0·1M-tris–acetic acid buffer, pH8·3; 30v/cm.; 180mA; rate of buffer flow in the cuvette, 50ml./hr.; sample introduction rate, approx. 2·2ml./hr.; passage time, about 5 days; sample introduction point, as indicated by arrow). The extinction values of the contents of the 48 collection tubes were read at 280mμ in 1cm. cells.

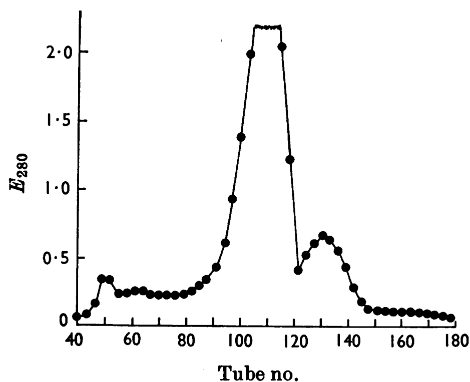

Fig. 2. Separation of proteins of kidney bean obtained by high-voltage electrophoresis on a Sephadex G-200 column (100 cm. × 4 cm.) equilibrated with 0·1 M-tris–acetic acid buffer, pH 8·3. The separated fractions (fraction size approx. 7·9 ml.) were read at 280 mμ in 1 cm. cells to locate the position of the proteins.

collected, concentrated by ultrafiltration and subjected to chromatography on a Sephadex G-200 column (Fig. 2). The two main protein components obtained were similar to those previously described (Pusztai, 1965c). The first and larger of the two components was eluted at 2·10 and the smaller at 2·62 void volumes (Fig. 2). The contents of tubes 96–122 and 123–147 respectively were pooled, concentrated by ultrafiltration and chromatographed on a Sephadex G-75 column (Figs. 3a and 3b). The first protein component was almost completely excluded from the gel bed; only a small amount of proteins of lower molecular weight was removed by this step (Fig. 3a). The second and smaller protein gave a major component at 1·76 void volumes together with smaller amounts of other protein components (Fig. 3b). In the final step of the purification each of the two main proteins was applied to a DEAE-Sephadex column and eluted with a gradient of sodium chloride in 0·05 M-tris–acetic acid buffer, pH 8·3. The first and larger protein component gave a near-symmetrical peak and was eluted between 0·26 M- and 0·32 M-sodium chloride concentration (Fig. 4a). A faint yellow pigment that stayed with the protein throughout the purification procedure was separated from the protein in this step; it was seen absorbed on the topmost part of the column and was not eluted under the conditions used. The second and smaller protein component was separated from a minor component and eluted from the DEAE-Sephadex column between 0·24 M- and 0·31 M-sodium chloride concentration. This protein, especially in concentrated solutions, had a pinkish-

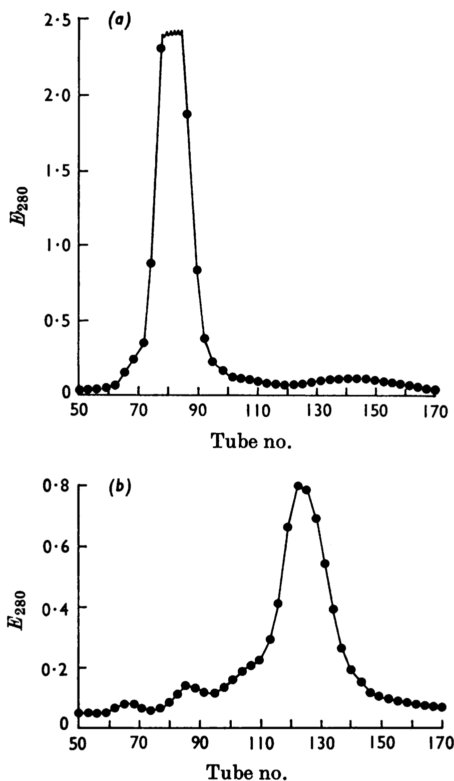

Fig. 3. Separation by chromatography on a Sephadex G-75 column (100 cm. × 4 cm.) of the protein fractions obtained from kidney bean and eluted at 2·1 void volumes (a) and 2·62 void volumes (b) from a Sephadex G-200 column (see Fig. 2). This column was also equilibrated and operated with 0·1 M-tris–acetic acid buffer, pH 8·3. The contents of the tubes (fraction size approx. 7·7 ml.) were read at 280 mμ in 1 cm. cells to locate proteins.

blue colour. The pigment, however, was not removed even by this last step in the purification. Both proteins obtained were shown to be homogeneous by the criteria employed (Pusztai, 1965c, and unpublished work) and these preparations were used for the analytical work. Portions of each protein were exhaustively dialysed against distilled water, freeze-dried and the samples weighed. From 130 g. of kidney bean the total yield calculated on this basis was 150 mg. of the major component, a glycoprotein containing 14·9% of N (Kjeldahl). This represented nearly 0·5% of the total seed N extracted. The yield of the smaller protein component was 72 mg. from 130 g. of kidney bean. This protein contained 15·4% of N and represented 0·23% of the total N extracted. The results of the chemical analyses for these two proteins are summarized in Tables 1 and 2.

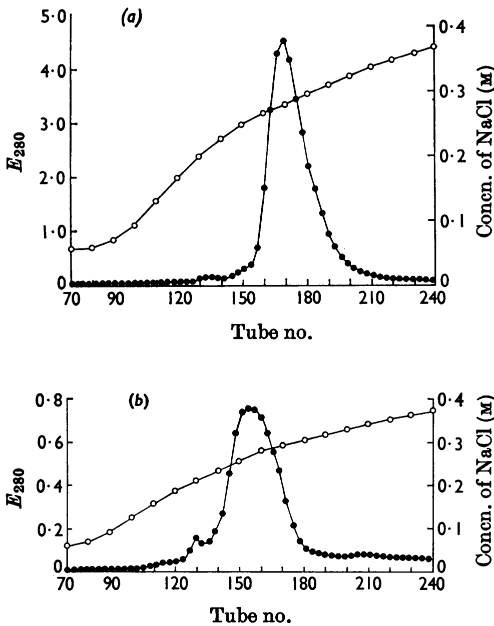

Fig. 4. Final step in the purification procedure of glycoprotein I (*a*) and the second and small protein component (*b*) from kidney bean: chromatography on a DEAE-Sephadex column (34 cm. × 4 cm.). The column was equilibrated with 0·05M-tris–acetic acid buffer, pH 8·3, containing NaCl (0·05M). The chromatogram was developed with a gradient of NaCl in the tris–acetic acid buffer. The concentration of NaCl in the eluted fractions (○) was determined by titration with standard $AgNO_3$ solution with K_2CrO_4 as indicator. The position of the proteins in the chromatogram was determined from the extinction values at 280 mμ in 1 cm. cells of the eluted fractions (●).

DISCUSSION

The present paper, as a part of a general examination of plant proteins, presents the full account of the isolation of two proteins from the seeds of kidney bean. Part of this work has already been described (Pusztai, 1965*c*). The isolation of the proteins was achieved by the application of well-accepted methods and these need no further comment.

The two proteins showed great contrasts in their chemical composition. The first and larger protein component, in accordance with previous results (Pusztai, 1965*c*, 1966), was a glycoprotein and was designated as glycoprotein I (Pusztai, 1966). The determination of the exact sugar composition of glycoproteins presents certain difficulties (e.g. Johansen *et al.* 1960; Neuberger, 1964). In this instance, however, there is little doubt that the major carbohydrate constituents of glycoprotein I were D-glucosamine and D-mannose. There is more uncertainty about the exact amounts of the small arabinose, xylose and fucose components that were also present and could not be removed for example by washing the precipitated protein with aqueous solutions of trichloroacetic acid. Similarly, extraction with phenol failed to decrease the amount of the total bound carbohydrate (Pusztai, 1966). The quantitative results of the various sugar analyses, however, should not be taken as the final values, as the distinction between the individual sugars, especially if they are present in highly disproportionate amounts, is not well defined in these analytical techniques. The quantitative paper-chromatographic method of Wilson (1959),

Table 1. *Results of carbohydrate estimations on proteins from kidney bean*

Experimental details are given in the text. The results of analyses are given in g. of constituent/100 g. of (*a*) original material, (*b*) material insoluble in aq. 5% (w/v) trichloroacetic acid and (*c*) material recovered from phenol. The second protein component was not subjected to treatment with trichloroacetic acid or phenol and the results were obtained on the original protein only.

	Glycoprotein I			Blue protein
	(*a*)	(*b*)	(*c*)	(*a*)
Total neutral sugar expressed as mannose*	11·7	11·4	11·8	0·2
Total neutral sugar expressed as mannose†	11·0	11·2	11·3	0·1
Mannose‡	7·8	7·8	7·9	0·1
Pentoses	3·2	3·2	3·2	0·1
Methylpentoses	0·8	0·7	0·8	0
Uronic acids	0	0	0	0
Glucosamine§	2·77	2·73	2·79	0

* Estimated from the colour developed with the phenol–H_2SO_4 reagent.

† Results of the orcinol–H_2SO_4 estimations.

‡ Determined at 440 mμ from the colour developed with the carbazole–H_2SO_4 reagent.

§ Estimated after hydrolysis with 4N-HCl at 100° for 4 hr. by chromatography on an ion-exchange resin column.

Table 2. *Analytical values for proteins of kidney bean*

Details of purification and methods of analyses are described in the text. The results of chemical analyses (average of five separate estimations) are expressed as g./100g. dry wt. of glycoprotein I or blue protein.

	Glycoprotein I	Blue protein
Asp	16·00	16·65
Thr*	7·83	7·13
Ser*	11·40	12·91
Glu	8·75	9·37
Pro	3·36	6·56
Gly	2·80	1·81
Ala	3·86	3·32
CyS†	0·06	14·45
Val	7·78	1·75
Met	1·16	1·48
Ile‡	4·82	4·79
Leu‡	5·40	3·40
Tyr	4·60	2·34
Phe	7·14	2·01
NH_3*	1·75	0·88
Lys	4·37	5·82
His	1·71	5·14
Arg	5·02	5·40
Tyr§	4·65	1·83
Trp§	3·27	1·55
N (Kjeldahl)	14·9	15·4
N (amide)	1·42	0·75
% of N accounted for	96·4	98·7

* Obtained by a linear extrapolation to 0hr. of the results of analyses on the 24hr., 48hr. and 72hr. hydrolysates.

† Results of analyses on performic acid-oxidized proteins.

‡ Results taken from the analyses of the 72hr. hydrolysate.

§ Obtained by the spectrophotometric method of Bencze & Schmid (1957).

clearly the most specific of the methods used, gave essentially similar results, although the amount of mannose (9·0%) found was higher than that (7·8–7·9%) obtained by the other techniques. The amount of fucose was difficult to estimate (about 0·2%) because of incomplete separation from xylose, and the combined amounts of arabinose (0·8%) and xylose (about 1·3%) also fell short of the total pentose values obtained (3·2%) with the cysteine–sulphuric acid method of Dische (1949). Only faint traces of glucose and galactose could be seen on the chromatograms.

The pinkish-blue smaller protein component, on the other hand, was almost entirely devoid of sugars (Table 1). The nature of the pigment has not yet been established, although it is firmly combined with the protein. There are clear differences also in the amino acid composition of the two proteins (Table 2). Glycoprotein I contained no or very little cyst(e)ine but was rich in aromatic amino acids. Although this sulphur-containing amino acid is very sensitive to degradation when heated with strong acids in the presence of carbohydrates, the recovery of less than 1 cysteic acid residue/protein unit of mol.wt. 60000 after oxidation with performic acid pointed to the possibility of this glycoprotein I genuinely lacking cyst(e)ine. The pinkish-blue protein, on the other hand, contained more than 14% of half-cystine but was relatively poor in aromatic amino acids and valine. The overall amino acid composition and, particularly, the high cystine and low valine content of the latter protein showed a strong resemblance to that of the trypsin inhibitors isolated from lima beans (Fraenkel-Conrat, Bean, Ducay & Olcott, 1952; Jones, Moore & Stein, 1963; Ferdinand, Moore & Stein, 1965) and from *Phaseolus aureus* (Chou & Chi, 1965). The protein, when tested by a method similar to that described by Ferdinand *et al.* (1965), inhibited the action of trypsin (A. Pusztai, unpublished work).

Finally, a comparison of the analytical values obtained for glycoprotein I in the present work with those described before (Pusztai, 1965*c*, 1966) shows a reasonable general agreement between the two sets of values. Although a few amino acids, notably lysine, were found to be decreased in amount, the results for most amino acids showed small increases, probably as a consequence of the removal of the yellow pigment in the last step of purification described in the present paper. This, taken together with the apparent homogeneity found by other methods, supports the high purity claimed for these two proteins.

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