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Fractionation of Proteins from Kidney Beans (*Phaseolus vulgaris*)

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Proteins extracted from the seeds of black kidney beans have been separated by ammonium sulfate fractionation and free-flow electrophoresis into fractions: two soluble in salt solutions and nine water-soluble. Four of these fractions had hemagglutinating activity, but only two different hemagglutinating proteins could be demonstrated with reasonable certainty. Amino acids and sugars were determined in all fractions; rhamnose, fucose, galactose, xylose, mannose, arabinose, glucose, an amino sugar, and one unidentified reducing compound were detected in one or several of the hydrolyzed proteins. Immunological cross reactions could be detected by a double-diffusion technique between the different hemagglutinating fractions as well as between the inactive fractions. Six different precipitation lines were observed when the water-soluble proteins containing the six fraction, which precipitate with ammonium sulfate, were submitted to immunoelectrophoresis. Similar extracts from red or white varieties of *Phaseolus vulgaris* gave very different patterns.

INTRODUCTION

Bean proteins have been the object of various investigations since Osborne's first studies (1). It was found that the extractable proteins are mostly globulins, a fact later emphasized by several other authors. Some considered that there were two globulins present (2) while others found three (3–5). Special interest was aroused by the fact, first observed by Landsteiner and Raubitschek (6), that beans contain a soluble hemagglutinin. This has been shown to be a protein, and several authors have isolated active fractions which they considered to be pure (7–9). These latter data, however, could not be related to the earlier investigations because nothing was known about the number of fractions in bean seeds active or inactive as hemagglutinins, or their distribution between globulins and albumins.

In the present study we have obtained new information on the number of proteins present in beans, their chemical composition, and their biological activities.

EXPERIMENTAL

Black kidney beans (*Phaseolus vulgaris*), acquired in Caracas, were dried and finely ground. The soluble proteins were fractionated as summarized in Diagram 1. One-kg lots of the meal were extracted overnight at 4°C with 5 liters each of a 1% NaCl solution, filtered through cloth, and centrifuged; the clear solutions were dialyzed against tap water. The precipitated globulins were separated by centrifugation, and the soluble proteins were precipitated by saturation with ammonium sulfate. The globulins were separated into two fractions, using the technique of Goa and Strid (10). After repeating this procedure three times, both fractions were dried by lyophilization; they were called fraction D (insoluble in 0.2 *M* NaCl, pH 4.5) and fraction E (soluble).

The ammonium sulfate-precipitated material was dissolved in water, dialyzed, adjusted to pH 6.0, and precipitated with ammonium sulfate at 55% and later at 70 and 100% saturation. All three fractions thus obtained were reprecipitated twice more, dialyzed against running water, then against distilled water, and lyophilized. In accordance with our previous work (8), we called the fraction precipitating with $(NH_4)_2SO_4$ at 55% saturation, fraction C; the next, fraction A; and the one precipitating last, fraction B.

A small amount of additional material has been obtained from the supernatant of the first ammonium sulfate precipitation by dialysis against distilled water; this was called fraction G.

Fraction A was usually homogenous on paper electrophoresis; in some cases it could be freed of

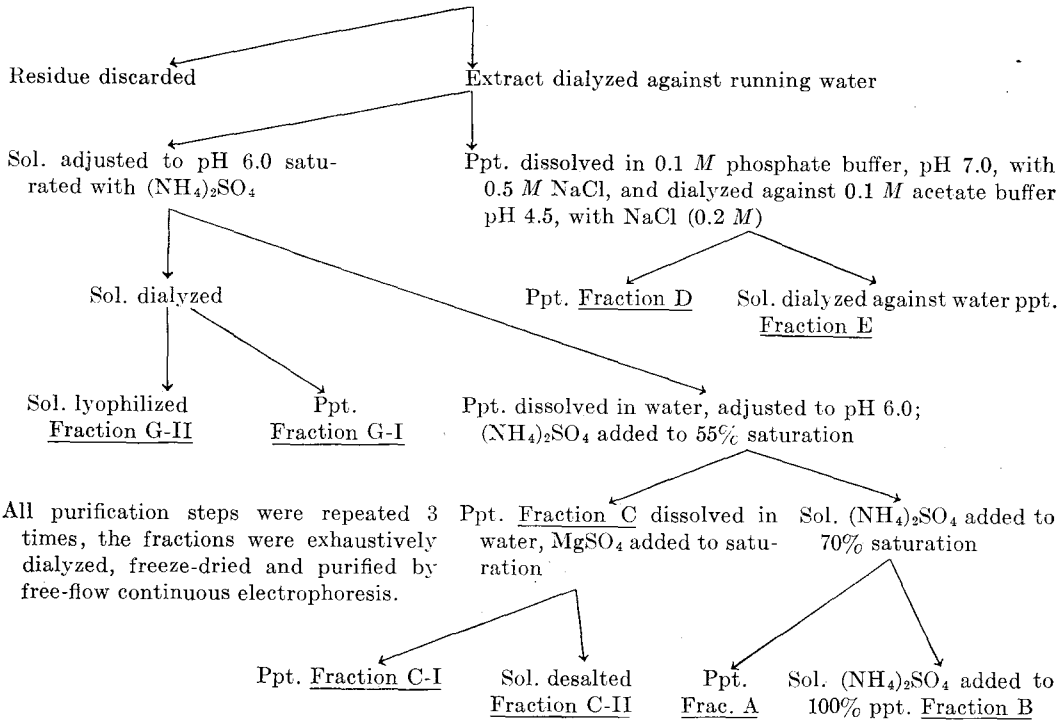

DIAGRAM 1. Steps involved in the fractionation of bean proteins (1 kg ground beans suspended in 5 liters 1% NaCl solution.

impurities by first precipitating at 0° with 30% alcohol, dissolving the precipitate in water, dialyzing the clear solution against distilled water, and precipitating again with $(NH_4)_2SO_4$ at 70% saturation.

In some preparations of fraction B, only two main proteins could be detected, while in others three more zones were apparent on paper or on free-flow electrophoresis. In the former case separation could be achieved by dissolving the fraction in 0.1 *M* sodium acetate and precipitating by adding 30% alcohol, after adjusting the pH to 4.8 with 0.1 *N* HCl. The precipitate was separated from the supernatant, and both were dialyzed and lyophilized.

Free-flow continuous electrophoresis of Hannig (11) was used for further purification except for fractions D and G. The protein content of the effluent fractions was determined by the method of Lowry *et al.* (12) in a Beckman DU spectrophotometer; the hemagglutination test was also performed.

A crystalline protein, which was called fraction F, was obtained from the ground bean seeds by the method described by Bourdillon (13).

The soluble fractions were submitted to paper electrophoresis (Elphor apparatus; Bender and Hobein, Munich, Germany) in pH 8.6 Veronal buffer. Four paper strips were used for each fraction; one was colored with amido black, the other was cut in 1-cm wide strips which were put separately into test tubes containing 1 ml of a 0.2% erythrocyte suspension, in order to study the hemagglutinating activity of the different proteins. Other strips were stained by the periodate-fuchsin-sulfite (PAS) method for the detection of glycoproteins (16) or with sudan black for lipoproteins (16).

The amino acids were determined in protein samples of about 2 mg. They were hydrolyzed with redistilled HCl in ampoules sealed under nitrogen at 100° for 24 hours, and the amino acids were analyzed with the automatic "Elphor" (Bender and Hobein, Munich, Germany) apparatus of Hannig (14). Tryptophan and cystine were determined separately by microbiological methods (15).

For the determination of the sugars, 5–10-mg samples were hydrolyzed with 2 *N* H_2SO_4 in a boiling water bath for 2 hours, cooled, neutralized with $Ba(OH)_2$, 9 times the volume of ethyl alcohol added, centrifuged, and the solution distilled under reduced pressure to dryness. The residue was dissolved in a small amount of water, transferred to a small test tube, and dried in a vacuum desiccator over P_2O_5. The residue was taken up

with 0.05 ml of water and aliquots were chromatographed 3 times on Whatman No. 1 filter paper by the ascending technique using butanol-acetic acid-water (4:1:5), phenol-water, or ethyl acetate-pyridine-water (2:1:2).

In other experiments, Dowex 50 × 8 acid form was used to achieve the liberation of the sugars present in the protein fractions. In this case, it was necessary to hydrolyze in a sealed ampoule under nitrogen for 3 days at 105°, but less sugar was sometimes observed in the samples thus prepared than in those hydrolyzed with sulfuric acid.

The following reagents or reactions were used to locate and identify the spots: $AgNO_3$ in acetone, aniline phatalate, naphthyl-resorcinol, acetyl-acetone and dimethylamino-benzaldehyde, the Waldron Rimini reaction (16), and glucose oxidase with dianisidine (17).

Quantitative analysis of the sugars were performed in the hydrolyzed fractions after separation by paper chromatography. The spots, revealed upon coloration with triphenyl tetrazol, were eluted with methanol acetic acid, and the extinction of the eluates was estimated in a Zeiss spectrophotometer model PMQ II at 578 mμ. Standard curves were prepared separately with each sugar which had been chromatographed under the same conditions.

Hemagglutination was studied with 0.2% suspensions of washed blood cells from mice or rabbits. Both cell preparations proved to be equally sensitive. They were agglutinated by identical dilutions of the different active bean proteins. The details of the technique used have been described earlier (18).

RESULTS

Fractionation of Proteins

Paper electrophoresis of the total bean extract would not permit recognition of all the different components. The bulk of the proteins migrated as a broad band at a velocity similar to serum globulin. The hemagglutinating activity was found in this zone. (Fig. 1 and Table I).

All the zones stained with amido black were identified as glycoprotein by the PAS-staining technique. Only the slowly moving fractions, which gave positive hemagglutinin reactions, could be stained with sudan black under the experimental conditions, using the unfractionated soluble proteins; but the purified fraction B-β and F after paper electrophoresis could also be stained with this dye.

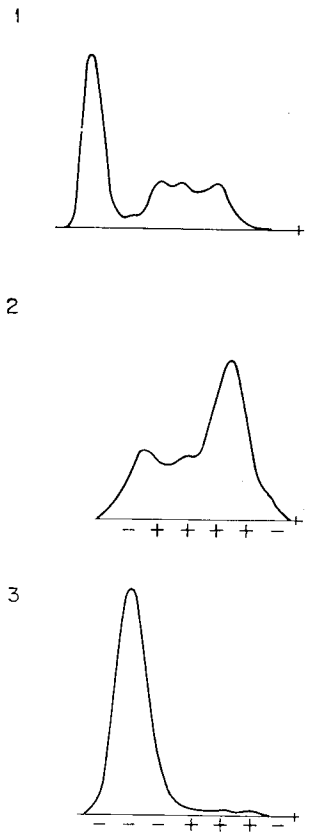

Fig. 1. Paper electrophoretic patterns of: (1) human blood serum; (2) water-soluble bean proteins; and (3) fraction E (salt-soluble bean protein). Veronal buffer, pH 8.6; μ 0.05; 220 V (8 V/cm); coloration with amido black. Hemagglutinating activities were determined separately with the eluates from 1-cm wide pieces of another paper strip subjected to the same electrophoretic procedure but not stained, and are indicated by + signs.

The yield of the water-insoluble fractions was about 90% of the total extracted protein. The three crude fractions A, B, and C were obtained in about equal amounts with some differences observed between different lots of seeds.

The water-soluble protein most easily isolated in a rather pure form is fraction A, which has been described earlier as phaseolotoxin A (8). After careful repeated fractionation with ammonium sulfate between 0.55 and 0.7 saturation, this protein gave one single zone on paper and on free-flow

TABLE I

PAPER ELECTROPHORETIC MOBILITY AND HEMAGGLUTINATING ACTIVITY OF BEAN PROTEIN FRACTIONS PURIFIED BY FREE-FLOW ELECTROPHORESIS

Fraction	Mobility as % of human serum albumin[a]	Hemagglutination, highest dil.
A	0.50	1:128.000
B-α	0.37	1:64.000
C-I-α	0.48	1:32.000
C-II	0.51	1:128.000
B-β	0.74	—
C-I-β	0.84	—
E	0.80	—
F	0.75	—

[a] Veronal buffer, pH 8.6

electrophoresis (Fig. 2) with an isoelectric point of pH 4.9. The molecular weight of this protein has been found to be about 128,000 (19), and its hemagglutinating and toxic properties have been described (18).

Fraction B showed considerable variations when obtained from different bean samples, as demonstrated by the results obtained with two samples separated by free-flow electrophoresis shown in Fig. 2 as B-old and B-new. Only the main component B-β and the active fraction B-α have been analyzed.

The hemagglutinating activity of B-α was quite unstable and disappeared completely after 4–6 months. Such a rapid loss of activity was observed in no other fraction.

Previously, a glycoprotein fraction containing about 30% of carbohydrate and active in the hemagglutination test was isolated from fraction B prepared from the lot of bean seeds which showed five peaks in the free electrophoresis experiment (B-old of Fig. 2) (20). This material could not be detected in other bean samples.

Fraction C-I gave two zones when submitted to free electrophoresis: C-I-α and C-I-β; the former agglutinated erythrocytes but the latter did not (Table I).

The water-insoluble fraction E, corresponding to vicellin of Osborne (1), showed some hemagglutinating activity which did not move with the same velocity as the main component in paper electrophoresis (Fig. 1). Free electrophoresis showed the presence of several differently moving proteins, but only the main fraction could be obtained in sufficient quantity for further studies. After repeated washings with distilled water, fraction E could be brought slowly into water solution and could be reprecipitated by traces of bi- or trivalent metal ions. The sedimentation constant of this fraction has been shown to vary with pH and ionic strength (2, 21). The hemagglutinating component could not be separated by dissolving it repeatedly in salt solution and precipitating by dialysis, but was completely separated by free-flow electrophoresis (Fig. 2). The amount of the fraction active in the hemagglutinin test was only about 5% of the total fraction E. Nevertheless, after separation by free-flow electrophoresis, the hemagglutinin activity of this fraction was only about twice that of the crude fraction E. Mixing both fractions again would not restore the hemagglutinating activity lost in the purification. Fraction D is similar to the legumin studied by Danielson (22).

The material in some of the bean extracts not precipitated by saturation with ammonium sulfate had a weak hemagglutinating activity, which was not destroyed by boiling for 20 minutes; this procedure destroyed the blood agglutinating action of all the other fractions studied. This heat stable activity was detectable only in one of six lots of beans assayed, and this single lot was too small for isolation of the active principle. A similar heat-stable hemagglutinating action has been observed in potatoes (32).

Upon dialysis of the supernatant from the precipitation of a bean extract by saturation with $(NH_4)_2SO_4$, a precipitate formed which contained 2.5% N (micro-Kjeldahl) and gave strongly positive reactions for organic P and Fe. It was called G-I. The filtered solution yielded fraction G-II after lyophilization. This fraction contained only 2.3% N (micro-Kjeldahl). It was assayed for hemagglutinating as well as for trypsin inhibitor activity (23) with negative results in both cases. Both fractions G were soluble in 5% tricloroacetic acid.

Four of the bean protein fractions were active as hemagglutinins while all the others were not (Table I). Between the active frac-

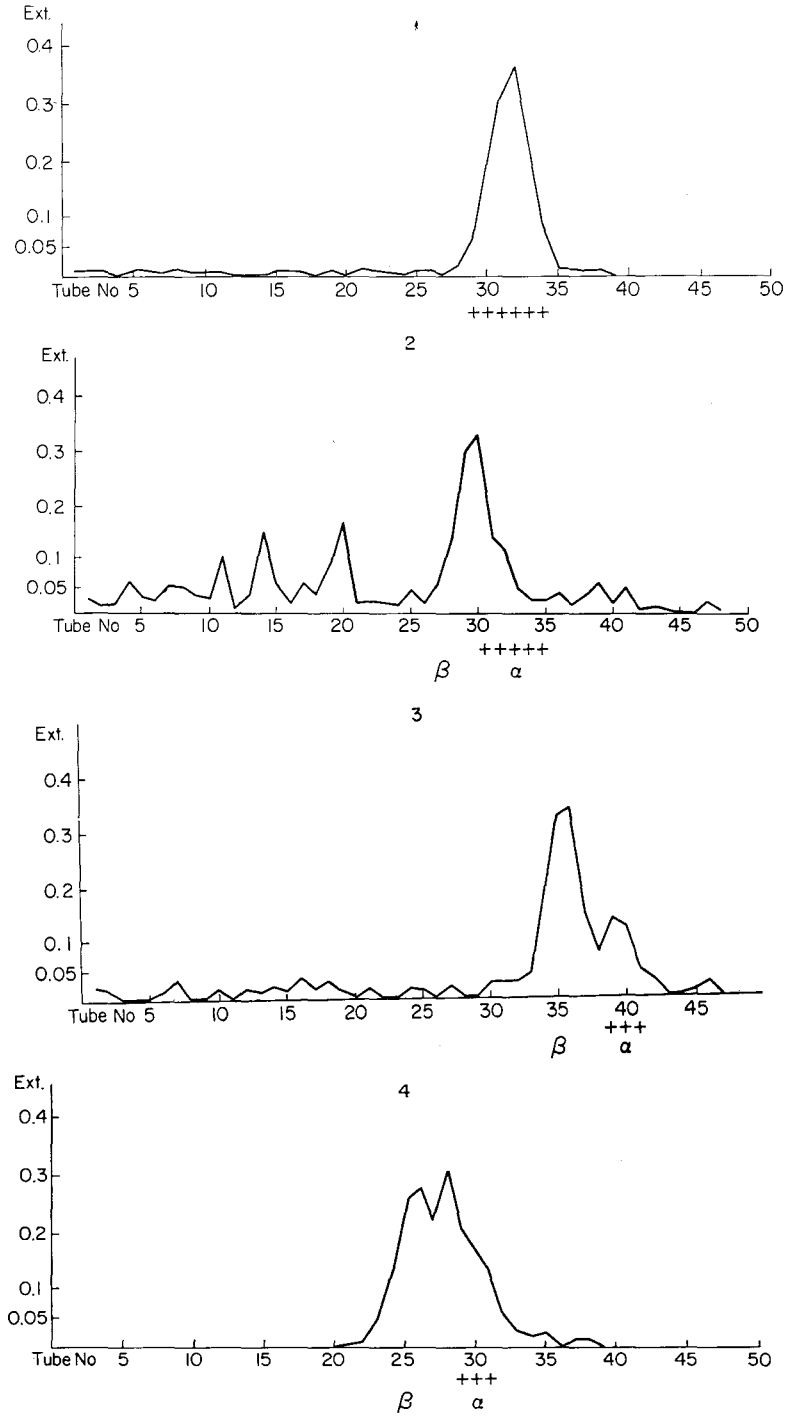

FIG. 2. Free-flow continuous electrophoresis of different fractions of bean proteins obtained by procedure of Diagram. 1. Veronal buffer, pH 8.6; μ 0.03; 1600 V, 160 mA (35 V/cm). The protein concentration was determined colorimetrically at 750 mμ by the method of Lowry *et al.* (12). Aliquots were tested for hemagglutinating activity. No. 1 = Fraction A, No. 2 = Fraction B old, No. 3 = Fraction B new, No. 4 = Fraction C-I, No. 5 = Fraction C-II, No. 6 = Fraction E.

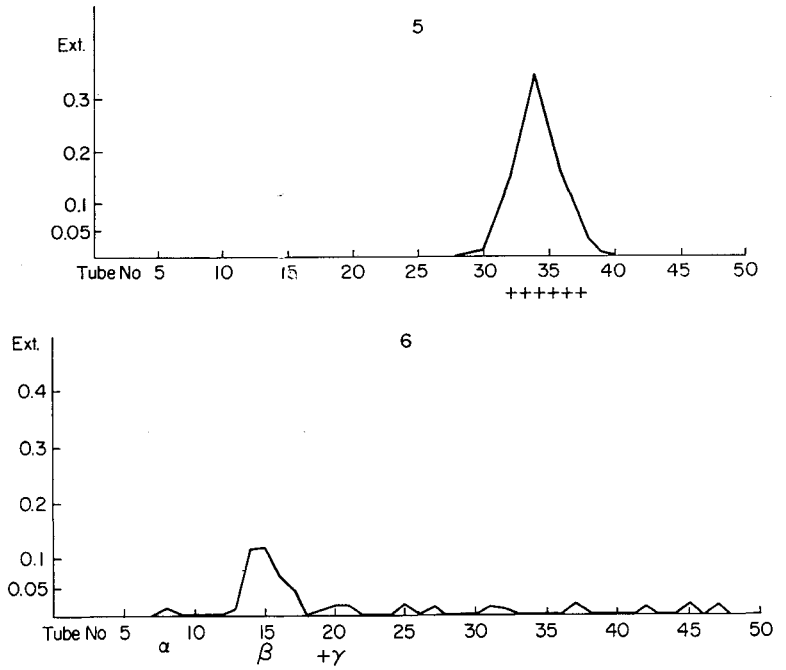

FIG. 2. *Continued*

tions there were some quantitative differences in their ability to agglutinate blood cells (Table I). Results concerning the activity against erythrocytes of different animals will be published separately. It may be mentioned that no specificity against human blood from persons of different blood groups was found in any of the fractions studied.

Both the water- and the saline-soluble protein fractions were very resistant to hydrolysis by trypsin or papain. Even after 24 hours of contact with a solution of one of these enzymes, very little ninhydrin-positive material was observed after precipitation with trichloro-acetic acid. Heat denaturation or working in the presence of 6 *M* urea would not alter these results significantly.

ANALYTICAL RESULTS

The amino acid compositions of the protein fractions are presented in Table II, and are expressed as moles of each amino acid per 100 moles of all amino acids (Mol %). The most abundant amino acids in the different fractions were: aspartic acid, glutamic acid, or serine, respectively. Important differences were found in the relative amounts of cystine, histidine, methionine, and tryptophan. In fraction A, in contrast to all other fractions, the quantity of leucine present was smaller than that of isoleucine. No proline could be found in fraction G-II.

The amino acid composition of fractions B-β and F was so similar that the small differences observed may be well within the limits of the experimental deviation. Small amounts of hydroxylysine have been found in two of the fractions. This amino acid has been detected recently in other plant proteins (24).

All the analyzed fractions differed in respect to the quantity of sugars they contain. Five different sugars were present in most of them, namely, fucose, xylose, mannose, galactose, and an amino sugar. Rhamnose and arabinose were found only in fractions C-I and G-II (Table III). All these sugars, with the exception of galactose, have not been found previously in beans. Evidence for the presence of glucose was found by the glucose oxidase test in several fractions. The amino sugar was detected by the Morgan-Elson reaction (16) but was not identified.

One additional faint spot was revealed by silver coloration in hydrolyzates of fractions

TABLE II

AMINO ACID CONTENT OF DIFFERENT BEAN PROTEIN FRACTIONS

(Expressed as mol%)

	A	B-α	B-β	C-I-α	C-I-β	C-II	D	E	F	G-II
Asp	13.60	12.50	12.72	12.63	14.86	15.28	10.77	14.48	11.80	11.50
Glu	6.81	9.00	15.88	7.02	6.72	7.49	11.63	14.85	16.23	11.78
Thr	8.55	5.41	4.06	7.90	8.04	9 39	5.20	4.18	4.15	5.21
Ser	10.37	13.31	11.61	14.30	17.81	11.71	7.90	7.87	11.02	7.18
Pro	4.83	4.28	4.83	5.57	4.34	4.33	5.45	4.48	4.71	0
Gly	9.83	7.95	6.15	6.25	4.68	6.82	7.09	5.87	6.90	11.99
Ala	6.80	8.91	5.19	6.71	5.17	6.00	7.30	5.08	5.01	12.11
Cyst	0.51	0	0.16	4.23	1.18	0.67	0.39	0.04	0.13	0.10
Val	6.75	5.65	4.25	5.00	6.77	6.42	6.60	5.72	4.54	6.48
Met	0.10	0	0.79	0.10	0.71	0.12	1.34	0.90	0.81	1.03
Ileu	7.59	4.19	4.00	4.17	4.42	4.62	5.22	4.29	4.01	3.93
Leu	4.01	7.26	7.89	6.81	4.96	7.93	9.05	9.58	8.19	7.48
Try	2.77	4.12	2.90	2.93	3.56	2.25	3.10	2.95	2.68	2.44
Phe	5.90	6.75	5.11	4.29	5.04	6.24	5.51	5.86	4.91	2.77
His	0.92	1.60	2.72	1.58	2.07	0.90	2.68	2.85	3.05	1.12
Lys	4.61	5.46	6.88	5.59	4.49	4.22	5.49	6.87	6.40	6.53
Hydroxyls	0.85	0.39	—	—	—	—	—	—	—	—
Arg	3.19	2.85	4.41	3.14	4.18	3.37	4.88	3.68	4.67	8.19
Try	2.07	0.47	0.44	1.68	1.00	2.24	0.40	0.38	0.35	0.21

TABLE III

SUGARS IN BEAN PROTEINS

Sugars[c]	Fraction									
	A	B-α	B-β	C-I-α	C-I-β	C-II	D	E	F	G-II
Rhamnose	—	—	—	2.9	—	—	—	—	—	7.5
Fucose	7.1	19.2	10.0	3.8	6.1	9.3	—	—	11.4	7.9
Xylose	10.0	34.2	20.1	7.8	8.7	9.1	7.9	—	6.0	11.5
Arabinose	—	—		7.3	—	—	—	—	—	20.3
Mannose	61.6	33.8	54.5	55.8	71.4	67.6	70.0	78.6	73.9	16.3
Galactose + glucose	9.6	7.8	8.1	11.4	10.2	14.0	16.6	14.7	—	33.9
Glucosamine[a]	11.7	5.0	7.3	10.6	3.6	—	5.5	6.7	8.7	2.6
Total sugar content[b]	5.7	4.0	3.3	10.4	2.0	3.6	1.7	2.5	2.4	22.1

[a] Unidentified amino sugar determined as glucosamine.

[b] The amount of each sugar is given as percentage of the total sugars of each fraction; an unidentified rapid sugar compound present in fractions A and C-II has not been included.

[c] The sugars were separated by paper chromatography from hydrolyzates of bean protein fractions after 2 hours hydrolysis with 2 N H_2SO_4 at 100°, and were determined by colorimetry of the eluates of the spots colored with triphenyl tetrazol as described in the text.

A and C-II. The Waldron Rimini reaction for methylpentoses was negative. The unidentified spot traveled faster than rhamnose and slightly faster than desoxyribose when the chromatogram was developed with butanol- acetic acid-water (Fig. 3). Triphenyl tetrazol did not give a coloration at the corresponding site.

The highest carbohydrate content was that of fractions G-II with 22.1% and of

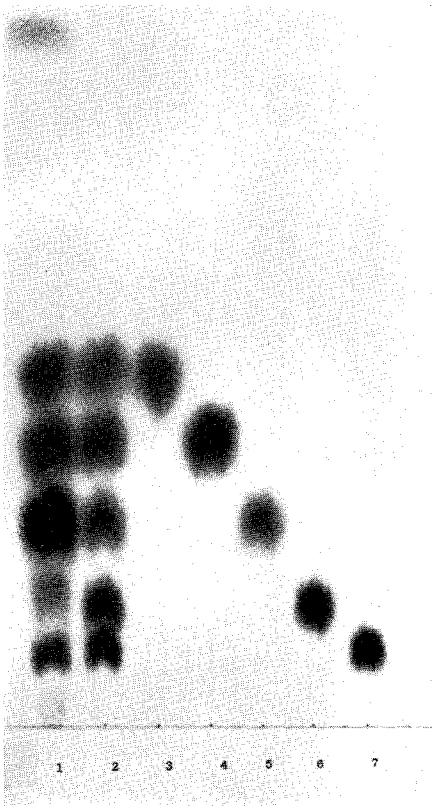

FIG. 3. Paper chromatogram of the hydrolyzates of fraction A heated for 2 hours with 2 N H_2SO_4. Solvent: butanol-acetic acid-water (4:1:5); stained with silver nitrate. No. 1: Protein hydrolyzate from 2.5 mg of fraction A; No. 2: mixture of sugars; No. 3: fucose, 5 μg; No. 4: Xylose, 5 μg; No. 5: mannose, 10 μg; No. 6: galactose, 5 μg; No. 7: glucosamine 10 μg.

C-I-α with 10.4%. In a previously studied lot of kidney beans, however, a fraction with about 30% of sugars was found (20).

IMMUNOLOGICAL EXPERIMENTS

Fifteen rabbits were immunized with the proteins described in the present paper. Six intravenous injections of 2 mg each were applied to the animals in the course of 3 weeks. Two weeks later they were bled and the sera were separated. Three more blood samples were obtained in the course of the following 3 weeks.

The activities of the immune sera were investigated by the double-diffusion technique of Ouchterlony (25). The antisera were used

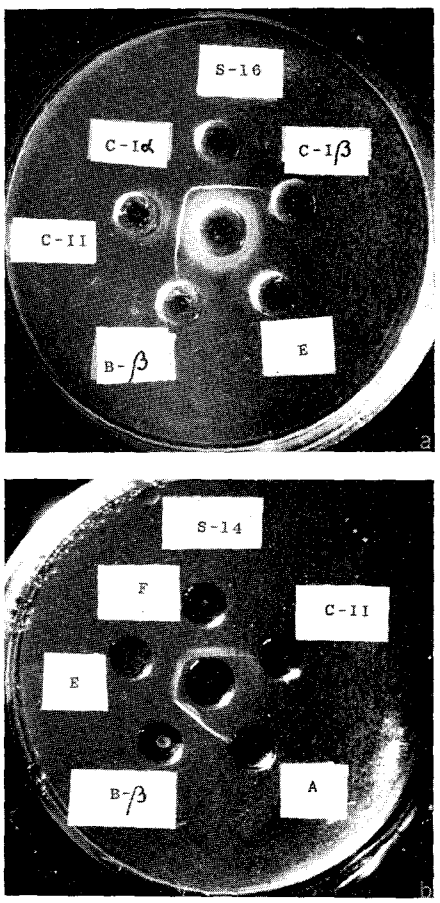

FIG. 4. Ouchterlony double-diffusion tests. Center well No. 16 contains rabbit antiserum to fraction A (active hemagglutinin) and No. 14 to fraction B-β (without hemagglutinating activity). The other wells contain the appropriately labeled bean protein fractions. Diffusion allowed to proceed for 7 days at 4°.

without dilution and the antigens were prepared for this test in a 0.1% concentration in physiological saline. In Fig. 4, the results of an experiment with the serum of a rabbit immunized against fraction A, showing fusing precipitation lines with other hemagglutinating fractions, are presented together with a plaque loaded with serum of a rabbit immunized against the inactive fraction E.

Cross reactions between the hemagglutinating and the nonhemagglutinating fractions were evident (Fig. 4). A detailed description of these experiments will be re-

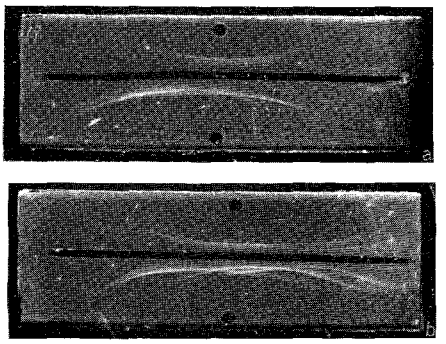

FIG. 5. (*a*) Immunoelectrophoresis of fractions A and B-α in agar gel, Veronal buffer pH 8.6, μ 0.1, 6 hours at 100 V and 4°C. In the central slot: antiserum from a rabbit sensitized against the water-soluble proteins from black beans was added, and diffusion was allowed to proceed for 2 days at 4°. (*b*) Immunoelectrophoresis of water-soluble, ammonium-sulfate precipitated proteins from black beans (above) and red kidney beans (below) performed under the same experimental conditions.

ported later. Danielson also observed that sera of rabbits immunized with one of the globulin fractions from beans would precipitate a second protein fraction isolated by him (22).

Three more rabbits were immunized with 6 intramuscular injections of 5 mg each of the water-soluble proteins from black beans, precipitated by saturation with ammonium sulfate and, after repeating this procedure, dissolved in 1 ml of physiological saline and emulsified with 1 ml of paraffin oil using Atlox R56 (Atlas Powder Comp. Wilmington, Delaware) as an emulsifier. Immunoelectrophoresis (37) on microscope slides was performed with the water-soluble black bean proteins and with a protein fraction obtained by the same procedure from red kidney beans. Six precipitation lines were observed with the former, while only three appeared with the latter when allowed to react with the serum obtained from the blood of the rabbits injected with the soluble black bean proteins (Fig. 5).

In immunolectrophoretic analysis experiments with the fractions separated by free flow electrophoresis and antiserum of rabbits sensitized with the unfractionated water-soluble proteins, single lines were observed with fractions B-β, E, and F, while the other fractions gave rise to two or three precipitation lines. The lines corresponding to B-α were not observed with purified fraction A (Fig. 5). The lines produced with fractions B-β and F were undistinguishable.

In order to obtain independent confirmation about the number of hemagglutinating fractions in beans, absorbtion experiments were performed by the following method: The washed cells of 1 liter of human blood were hemolyzed with distilled water and centrifuged, and the stroma were washed until the supernatant was completely colorless. To the centrifuged and moist material, 30 mg of the water-soluble bean proteins or of fraction A, dissolved in 1 ml of saline, was added and was left standing at 4° for 18 hours. The suspension was then centrifuged, and the stroma were washed twice with 1 ml water each time.

After repeating this procedure once more, the mixed supernatants and washings of each fraction were lyophilized and dissolved in 3 ml of water. No hemagglutinating activity could be detected. Nevertheless, the same precipitation lines were observed with these solutions when tested by the method of immunoelectrophoresis, as with the corresponding unabsorbed fractions.

In another experiment a sample of a 3-year-old fraction A, which had lost most of its hemagglutinating activity, was submitted to immunoelectrophoresis. The same precipitation lines were observed as with a fresh and active preparation.

DISCUSSION

Eleven fractions have been separated from crude bean extracts; ten were obtained by the fractionation method summarized in Diagram I, and one (fraction F) as a crystalline protein, using the procedure of Bourdillon (13). In this latter method dilute hydrochloric acid is used as an extractant.

The two fractions G-I and G-II, which are not precipitated by saturation with ammonium sulfate or 5% trichloroacetic acid, and which have a low nitrogen content, are not typical proteins, and their detailed study has not been included in the present work. Of all the protein fractions obtained, four

were active as hemagglutinins. The pattern of the paper electrophoretic analysis of the original salt free extract, as shown in Fig. 1, would not allow the detection of these different fractions. Electrophoretic mobility of several of these proteins, especially those active as hemagglutinins, are so similar that this method alone would not easily permit complete separation. The combination of classical fractionation methods with free-flow electrophoresis gave more satisfactory results.

Fraction C-II was similar in electrophoretic mobility and hemagglutinating activity to fraction A (Table I). Only minor differences between these two fractions were observed in amino acid and sugar content; they may not be different proteins.

Fractions A and B-α, both active hemagglutinins, differed significantly in respect to electrophoretic mobility, stability of hemagglutinating power, composition in sugars and amino acids, and especially in the immunoelectrophoretic behavior, as shown in Fig. 5; they are most probably different proteins.

A comparison between fraction B-β and the crystalline fraction F shows that the latter has about 1% carbohydrate less than the former, and that this is due to its lack of galactose and its reduced xylose content. As the two proteins have practically the same amino acid composition (Table II) and very similar electrophoretic mobilities (Table I), it seems possible that fraction F arises from fraction B-β through the extraction with hydrochloric acid. Rigas and Osgood (7) found a bean glycoprotein which lost most of its carbohydrate content by acid extraction.

Six of the fractions were soluble in water and precipitated by saturation with ammonium sulfate (Diagram I). Six precipitation lines could be detected by the immunoelectrophoretic technique with this crude fraction of water-soluble, ammonium sulfate precipitated proteins as shown in Fig. 5.

Prager and Speer (38) found three hemagglutinating fractions in bean extracts, but Punnett and Punnett (39) claim to have observed five. Our results would not allow a definite statement to be made on the number of different proteins in our black beans active as hemagglutinins. The chemical and immunological differences between fractions A and B-α indicate that there are at least two. The failure of the attempt to demonstrate the number of different hemagglutinating proteins by the combined procedures of absorbtion on stroma and immunoelectrophoresis of the unabsorbed portion was probably due to the fact that part of the agglutinins had lost their capacity to combine with blood cells without losing their immunological characteristics.

Fractions D and E are globulins according to their insolubility in water, the electrophoretic mobility of the latter was greater than that of all the others and was similar to human serum albumin. All the hemagglutinating fractions moved more slowly than the inactive proteins.

Several authors have observed that fractions of bean proteins separated by paper electrophoresis give positive carbohydrate reactions (5, 26–28) or have shown the presence of carbohydrates directly (7). However, in no case have the sugars present in bean proteins been identified.

Most of the sugars found by us in the hydrolyzates of isolated proteins are not present in the alcohol-soluble extract from beans in which only glucose, fructose, and polysacharides containing galactose besides glucose and fructose have been found (29). Fructose could not be detected in any of the hydrolyzates studied by us. Fucose, which is present in all the protein fractions, has been found only once in a legume, namely, in a hemicellulose of peas (30).

Experimental data on sugars in hydrolyzates from plant proteins are scarce. Waldschmidt-Leitz and Hochstrasser (24) found xylose, glucose, and arabinose in barley proteins, and Liener has shown that a hemagglutinating protein from soya contains glucosamine (31). Krüpe and Ensgräber detected xylose, arabinose, galactose, and an unidentified sugar in a phytoagglutinin from potatoes (32): fucose, xylose, mannose, and an amino sugar have been found in ricin (42).

The detection of bean protein fractions stained by sudan black confirms a previous observation of Ghetie (41). Recently, it was shown by paper electrophoresis that only

the toxic and hemagglutinating fraction from ricinus seeds can be stained with this dye (42). In beans, however, both hemagglutinating and inactive fractions could be stained in this way.

Beans have been shown to contain not only hemagglutinins but also protein fractions with toxic properties (18) and fractions active in stimulating the growth of the leucocytes in tissue culture (33). Only those fractions shown to possess hemagglutinating activity in the present work had toxic effects when injected into mice (34), and also stimulated the reproduction of lymphocytes from human blood *in vitro* (35), when the technique of Favier *et al.* (36) was used.

After absorbtion on stroma of human erythrocytes, both the hemagglutinating and the mitogenic activities of the active fractions disappeared. This observation is different from the results of Borjeson *et al.* (40), who claim to have obtained a mitogenic fraction from beans free of hemagglutinin. As Punnett and Punnett (39) state, the growth factor and phytohemagglutinin may be separable portions of one macromolecule. This could explain why, in our experiments, it was not possible to separate the two activities, as our fractionation procedure does not involve exposure to acid and alcohol as as the one used by the Swedish authors.

Some 12 samples of black kidney beans were extracted. The differences found between one sample and another were those of fraction B, as shown in Fig. 2, and of the hemagglutinating activity of the supernatant from the ammonium sulfate precipitation as mentioned. One sample of white beans, and two samples of red kidney beans, when compared with a sample from black beans, gave very different patterns in immunoelectrophoresis (Fig. 5). It can threfore be expected that the fractions found by us in black kidney beans are not present in all varieties of *Phaseolus vulgaris*.

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