ISOLATION AND PROPERTIES OF PROTEIN FRACTIONS FROM NAVY BEANS (PHASEOLUS VULGARIS) WHICH INHIBIT GROWTH OF RATS

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SUMMARY

A protein fraction which inhibits growth of weanling rats was obtained from Phaseolus vulgaris seeds of the Sanilac variety by extraction with sodium chloride solution and removal of extraneous materials by precipitation at pH 4.0 and absorption on 1:1 bentonite-Celite. The protein was precipitated by 0.75 saturation with ammonium sulfate. The growth inhibiting protein fraction contained at least four different proteins separable by electrophoresis, ultracentrifugation, immunodiffusion, and gel chromatography. Only one of the soluble proteins inhibited rat growth, and it comprised about 35% of the total growth inhibiting fraction and had hemagglutinating activity, stimulated leucocytes, contained very little trypsin inhibitor activity, and had an isoelectric point of pH 5.2 and a mol. wt of approx. 110,000. A protein which was insoluble at pH 4 and collected as a precipitate also inhibited growth of rats but because of the small amount isolated and its insolubility, it was not further studied.

INTRODUCTION

Kakade and Evans using a procedure described by Honavar et al. isolated five protein fractions from raw navy beans. Trypsin inhibitor activity was concentrated in fraction 3 which contained no hemagglutinating activity and depressed growth of rats. Most of the hemagglutinating activity was concentrated in fraction 5 which also depressed growth and contained some trypsin inhibitor activity. Fraction 4 caused the greatest growth inhibition in rats of any of the fractions when it was fed at a level that supplied very low levels of trypsin inhibitor or hemagglutinin activity.

Fraction 4 is not a single protein but a mixture of proteins. The present investi-
gation was made to isolate the active growth inhibitor from fraction 4, to see if the growth inhibiting activity can be separated from the hemagglutinating activity, and to determine some of the properties of the proteins of fraction 4.

**EXPERIMENTAL PROCEDURE**

Fraction 4 was isolated from finely ground seeds of Sanilac beans (*Phaseolus vulgaris*) by the procedure described by Kakade and Evans. The procedure was essentially as follows. 1 kg of ground beans was extracted with 10 l of 1% NaCl solution, and the extract was decanted and centrifuged. The supernatant was adjusted to pH 4.0 and the precipitate was removed by centrifugation and discarded. Then 100 g of a 1:1 (w/w) mixture of bentonite–Celite were added, the mixture was centrifuged, and the residue was discarded. The supernatant was made 0.75 saturated with ammonium sulfate and centrifuged. The supernatant was discarded and the residue was dialyzed against distilled water and lyophilized to give fraction 4.

Polyacrylamide gel electrophoresis was performed in a vertical watercooled cell at pH 9.2 using a Tris–EDTA–boric acid buffer according to the procedure of Peterson and at pH 2.2 using a glacial acetic acid–formic acid buffer according to the procedure of Peterson and Kopfler.

High-voltage electrophoresis in free-flowing buffer films was carried out in an apparatus (made by Dr Bender and Dr Hobein, Munich, Germany) described by Hannig with a few modifications. Several buffer systems were tried. The conditions used for the separations were a cuvette buffer of 0.03 M sodium acetate, pH 4.0 with a protein concentration of 1%, a sample introduction rate of 1.5 ml per h, a current of 200 mA at 1600 V, and 8 °C air temperature.

Phoroslide electrophoresis was performed using the special Millipore Corporation equipment, slides, and procedure. A 0.2 M veronal–HCl buffer, pH 6.8, was used in most of the experiments.

Analytical ultracentrifugation was performed in a Spinco Model E analytical ultracentrifuge at 59 780 rev./min and 20 °C in a standard double sector 12 mm cell as described by Pusztai and Watt. Buffers used were 0.1 Tris–0.05 M acetic acid containing 0.1 M NaCl of pH 8.3 and citrate–phosphate buffer of pH 3.04.

Trypsin inhibitor content of fraction 4 was determined by titration of acid released from TAME (p-tosyl-L-arginine methyl ester–HCl) by trypsin and by mixtures of trypsin and bean fraction 4 or pure isolated trypsin inhibitor in the Radiometer pH-Stat.

Immunodiffusion studies were made by the Ouchterlony method between an antiserum to dry bean seed extract and extracts of whole dry bean seeds and bean protein fraction 4.

Electrofocusing of bean seed protein fraction 4 was done in an LKB 110 ml column with pH 3–10 Ampholine or pH 5–8 Ampholine as described by Haglund.

Bean seed protein fraction 4 was also fractionated by gel chromatography at 4 °C through a 2.5 cm × 90 cm column of Sephadex G-200 equilibrated with a buffer (pH 5.5) composed of 0.1 M potassium acetate and 0.01 M EDTA. Elution was with the same buffer at a rate of 20 ml per h using an LKB peristaltic pump and monitoring at 280 nm with a Beckman Model DB spectrophotometer connected with a Sargent SRL recorder. Molecular weights of the fractions were estimated using
Blue Dextran (mol. wt 2000 000), γ-globulin (mol. wt 156 000), serum albumin (mol. wt 70 000), and ovalbumin (mol. wt 45 000) as standards.

Portions of proteins separated by Sephadex gel chromatography were hydrolyzed with constant boiling hydrochloric acid in evacuated sealed tubes at 110 °C for 24 h. Amino acid composition of the hydrolysates were determined with the Technicon Amino Acid Analyzer using a procedure based on the Piez and Morris\textsuperscript{13} modification of the method of Spackman \textit{et al.}\textsuperscript{14}.

The lymphocyte stimulating activity of the proteins was measured by [\textsuperscript{3}H]-thymidine incorporation into DNA of pig blood which had been enriched for lymphocytes. The final red cell-lymphocyte ratio obtained was 4:1. The proteins were tested at approximately 50 to 100 μg levels.

Hemagglutination was assessed by subjective observation of red cell clumping under the microscope using red blood cells from pig blood, and was only roughly quantitative.

RESULTS

That bean protein fraction 4 is heterogeneous was demonstrated by electro-

![Polyacrylamide gel electrophoresis of bean protein fraction 4. Electrophoresis was at pH 2.2 using a glacial acetic acid-formic acid buffer. The samples were applied on the line at the top of each strip. The four strips from left to right are decreasing levels of bean protein fraction 4.](image-url)
phoretic, ultracentrifugation, immunodiffusion, and gel chromatographic procedures. Polyacrylamide gel electrophoresis at pH 9.2 separated fraction 4 into two or three diffuse, poorly separated bands, but polyacrylamide gel electrophoresis at pH 2.2 separated it into four relatively strong bands and some weaker bands (Fig. 1). PhoroSlide electrophoresis at pH 6.8 separated bean protein fraction 4 into four distinct bands (Fig. 2). Four lines were also observed in the immunodiffusion experiment.

Analytical ultracentrifugation at pH 8.3 gave one major peak of $s_{20} = 6.2S$
comprising about 77% of bean protein fraction 4 and three minor peaks of $s_{20} = 9.1S$, $s_{20} = 3.2S$, and $s_{20} = 2.0S$ (Fig. 3). However, at pH 3.04 a faster sedimenting protein of $s_{20} = 17.5S$ was observed. This protein comprised about 21% of bean protein fraction 4, and the $s_{20} = 6.4S$ fraction was reduced to 57%. Changes in some of the minor constituents were also observed.

TABLE I

Proteins obtained by high voltage free-flowing electrophoresis of bean protein fraction 4

<table>
<thead>
<tr>
<th>Protein</th>
<th>Weight (g)</th>
<th>% of total*</th>
<th>Lymphocyte stimulating activity** (cpm)</th>
<th>Hemagglutination***</th>
<th>Rat growth**** (g gained)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A</td>
<td>0.39</td>
<td>8.5</td>
<td>90</td>
<td>0</td>
<td>18.20</td>
</tr>
<tr>
<td>4B</td>
<td>0.35</td>
<td>7.6</td>
<td>88</td>
<td>0</td>
<td>19.22</td>
</tr>
<tr>
<td>4C</td>
<td>1.07</td>
<td>36.3</td>
<td>2723</td>
<td>4+</td>
<td>12.12</td>
</tr>
<tr>
<td>4D</td>
<td>2.01</td>
<td>43.7</td>
<td>138</td>
<td>2+</td>
<td>15.20</td>
</tr>
<tr>
<td>4 ppt.</td>
<td>0.18</td>
<td>3.9</td>
<td>84</td>
<td>+</td>
<td>13.16</td>
</tr>
</tbody>
</table>

* Percent of total of bean protein fraction 4 applied to the electrophoresis apparatus. The recovery was nearly quantitative. See text.

** Measured by [3H]thymidine incorporation into DNA of pig blood which had been enriched for lymphocytes. The proteins were tested at approximately 50 to 100 μg levels.

*** Subjective assessment of pig blood red cell clumping in cultures.

**** Two rats were fed each experimental diet for 4 days. The basal diet was composed of sucrose 30%, corn oil 6%, Hegstedt salt mixture 4%, vitamin mixture (Nutritional Biochemicals) 2%, casein 10%, and corn starch 48%. Each bean protein was added to the diet at a level proportional to the percentage in bean protein fraction 4 or 4A 0.08%, 4B 0.08%, 4C 0.36%, 4D 0.44% and 4 precipitate 0.04%. Rats and feed were weighed daily. Rats fed the basal diet gained 15 and 22 g and those fed raw beans to supply 10% protein gained -8 and -10 g. Rats fed bean protein fraction 4 at a 1.00% level gained 5 and 10 g.
Fig. 4. High-voltage electrophoresis of bean protein fraction 4 in free-flowing buffer film of pH 4.0 sodium acetate in the apparatus described by Hannig. Introduction of the sample was opposite tube No. 6. 50 ml of a 1% solution (0.5 g) of bean fraction 4 was introduced into the apparatus at a rate of 1.5 ml per h. After each separation experiment, absorbance of contents of the tubes were read at 280 nm. In all 5.0 g of bean protein fraction 4 were fractionated by the procedure. Protein 4A was isolated from contents of fraction Nos 9–14, Protein 4B No. 21–25, Protein 4C No. 26–36, and Protein 4D No. 38–44.

Fig. 5. Gel chromatography of bean protein fraction 4 through a Sephadex G-200 column. Elution was with pH 4.4 potassium acetate–EDTA buffer (0.1 M potassium acetate, 0.01 M EDTA). Protein 4–1 was isolated from material eluted at 29–47 ml, Protein 4–2 at 49–67 ml, Protein 4–3 at 68–78 ml, and Protein 4–4 at 79–90 ml.

Free flowing electrophoresis at pH 4.0 separated bean protein fraction 4 into four proteins designated as 4A, 4B, 4C, and 4D (Fig. 4). A precipitate or residue that was insoluble in the pH 4.0 buffer was removed by centrifugation of the bean fraction 4 solution before the sample was introduced into the Hannig electrophoresis apparatus. A number of electrophoretic runs were made to give sufficient material for further studies. Some properties of the proteins are presented in Table I. Each protein was dialyzed against distilled water and lyophilized to give a dry powder. Protein 4C exhibited the greatest amount of lymphocyte stimulating activity, hemagglutination activity, and rat growth inhibition, although the precipitate or residue also inhibited rat growth. The growth inhibition studies were not very conclusive because of variability in rat growth rates. Rats fed Protein 4C were the only rats fed a fraction where both rats gained less than the 15 g gained by one of the rats fed the control diet.

Gel chromatography of bean protein fraction 4 through a Sephadex G-200 column eluting with pH 4.4 potassium acetate–EDTA buffer separated the mixture into four distinct components designated as 4–1, 4–2, 4–3, and 4–4 (Fig. 5). Protein 4–1 was at times eluted as a large peak with three sub-peaks as indicated in the figure. However, each of the three portions of the peak only gave one band on PhoroSlide electrophoresis and that was the same for all three. PhoroSlide electrophoresis indicated that Sephadex G-200-separated Protein 4–1 and electrophoresis-separated Protein 4D are similar as are Sephadex-separated Protein 4–2 and electrophoresis-separated Protein 4C and Sephadex-separated Protein 4–3 and electrophoresis-separated Protein 4B (Fig. 2). Comparison of the elution volumes of the four proteins obtained by gel chromatography through Sephadex G-200 with those of reference proteins of known molecular weight indicated approximate mol. wt for Protein 4–1 of over 300 000, of Protein 4–2 of 110 000, of Protein 4–3 of 50 000, and of Protein 4–4 of 28 000. Amino acid contents of these proteins are given in Table II.
TABLE II

PROTEINS OBTAINED BY SEPHADEX G-200 CHROMATOGRAPHY OF BEAN PROTEIN FRACTION 4

Amino acid values are given as percentages.

<table>
<thead>
<tr>
<th>Protein</th>
<th>4-1</th>
<th>4-2</th>
<th>4-3</th>
<th>4-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoretic equivalent</td>
<td>4D</td>
<td>4C</td>
<td>4B</td>
<td></td>
</tr>
<tr>
<td>Percent of total*</td>
<td>51.0</td>
<td>31.1</td>
<td>9.6</td>
<td>8.4</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>13.0</td>
<td>15.1</td>
<td>15.5</td>
<td>12.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.5</td>
<td>7.8</td>
<td>7.4</td>
<td>6.4</td>
</tr>
<tr>
<td>Serine</td>
<td>6.5</td>
<td>8.7</td>
<td>9.4</td>
<td>7.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.3</td>
<td>10.0</td>
<td>9.7</td>
<td>8.8</td>
</tr>
<tr>
<td>Proline</td>
<td>3.5</td>
<td>3.7</td>
<td>3.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.3</td>
<td>4.0</td>
<td>4.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.3</td>
<td>4.5</td>
<td>3.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Valine</td>
<td>5.2</td>
<td>6.9</td>
<td>6.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.6</td>
<td>0.3</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.2</td>
<td>5.6</td>
<td>5.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.2</td>
<td>9.4</td>
<td>7.8</td>
<td>7.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.9</td>
<td>3.1</td>
<td>4.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.8</td>
<td>7.6</td>
<td>7.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.4</td>
<td>5.6</td>
<td>5.4</td>
<td>6.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.9</td>
<td>1.8</td>
<td>1.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.0</td>
<td>3.4</td>
<td>4.3</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Percent of total weight of fraction 4 isolated in the four fractions which was found in each of one of the fractions.

Compared to the amounts of protein needed for feeding studies, only small amounts of the four proteins were obtained by electrophoresis in free-flowing buffer films or by gel chromatography. A short time feeding procedure with two rats per group was investigated as an assay procedure for growth inhibition by the proteins. Studies with ground raw beans and isolated bean protein fraction 4 indicated that the procedures should be suitable for determining growth inhibition. Growth inhibition was observed with Protein 4C obtained by electrophoresis (Table I), and with the precipitate, but values obtained were not clear cut and growth inhibition by any protein was not nearly as great as that by fraction 4. Practically all of the lymphocyte

Fig. 6. Isoelectric focusing at pH 3–10 of bean protein fraction 4.

Fig. 7. Isoelectric focusing at pH 5–8 of bean protein 4C (left) and of bean protein 4-2 (right).
stimulating activity and the hemagglutinating activity of bean protein fraction 4 was found in Protein 4C. Bean protein fraction 4 contained only 0.6% of trypsin inhibitor.

Bean protein fraction 4 was separated into four or more proteins by isoelectric focusing with pH 3–10 Ampholine (Fig. 6). The four peaks had isoelectric points of approximately pH 4.3, pH 5.1, pH 5.7, and pH 6.4. Amounts of the proteins isolated by this procedure were insufficient for further studies. Bean Proteins 4C and 4-2, when subjected to isoelectric focusing in pH 5–8 Ampholine, gave peaks at pH 5.2, which can thus be assumed to be the isoelectric point of both (Fig. 7).

DISCUSSION

Raw navy beans (P. vulgaris) contain at least three proteins which are detrimental to animals consuming the raw beans. These are the trypsin inhibitors, the phytohemagglutinins, and the growth inhibitor. Stead et al. observed a separation of hemagglutinating activity and intraperitoneal toxicity by DEAE-cellulose chromatography of Natal Round Yellow Bean (P. vulgaris) extracts. Bean protein fraction 4 was shown by Kakade and Evans to contain both hemagglutinating and trypsin inhibitor activity, but at the levels that the various bean protein fractions were fed to rats bean protein fraction 4 supplied very little trypsin inhibitor activity or hemagglutination activity as compared to other fractions which caused much less inhibition of growth of weanling rats. The bean protein fraction 4 used in the present study contained less than 1% of trypsin inhibitor, so trypsin inhibition was not investigated for any of the subfractions.

High voltage free-flowing electrophoresis separated bean protein fraction 4 into four subfractions or proteins designated A, B, C, and D (Fig. 4). Protein 4A while exhibiting high ultraviolet absorption at 280 nm, comprised less than 10% of the total weight. Rat growth inhibitor activity appeared to be found almost exclusively in Protein 4C and in the precipitate. Protein 4C, which accounted for 36% of fraction 4, also contained most of the lymphocyte stimulating activity and the hemagglutinating activity of fraction 4. The data support the observation of Jaffe and Hannig that P. vulgaris seeds contain at least two hemagglutinins and that these phytohemagglutinins differ in growth inhibitory or toxic effects for the rat.

One cannot unequivocally interpret the rat growth inhibition studies because of the small number of rats used and the short time that they were kept on the experiment. Growth rates of the duplicate rats fed the basal diet and those fed the basal diet containing 1.00% of bean protein fraction 4 did not agree very well. One of the basal diet-fed rats gained 15 g and one of the rats fed bean fraction 4 gained 10 g. The difference was not large. Only Proteins 4C and the precipitate appeared to inhibit rat growth. A much lower level of the precipitate was fed than of Protein 4C, but the growth inhibition was not as definite as it was with Protein 4C.

At the present time we cannot be certain that bean Proteins 4C and 4-2 are similar and are single proteins. PhoroSlide electrophoresis patterns of bean Proteins 4C and 4-2 are similar but both are characterized by a doublet band or by two bands. Bean Protein 4C (4-2) could be composed of two proteins of similar molecular weight and electrophoretic mobility at pH 4.0 but that differ in electrophoretic mobility at pH 6.8. The isoelectric point obtained by isoelectric focusing was pH 5.2.
The pH 4.0 insoluble residue or precipitate of bean fraction 4 comprised 3.9% of the total fraction. The origin of the precipitate is unknown, but because it was growth-inhibitory, one could assume that it was formed from Protein 4C. Protein 4C could be a mixture of two proteins, a growth-inhibitor and a protein with lymphocyte stimulating activity, and part of the growth-inhibitor could have precipitated during electrophoresis. Another possibility is that Protein 4C is a dimer composed of dissimilar monomers and that it dissociates into two parts at pH 4.0, one having lymphocyte stimulating activity and the other growth inhibitory activity and some of the growth inhibitor precipitated. The third possibility is that a single protein exists but that on precipitation it lost its lymphocyte stimulating activity.

Ultracentrifugation studies of bean protein fraction 4 indicate that at pH 8.3 about 77% of the protein sedimented at $s_{20} = 6.25 S$, but that at pH 3.04 part of the protein (21% of total) aggregated to give a protein sedimenting at $s_{20} = 17.55 S$. Which of the proteins of bean protein fraction 4 is responsible for the pH dependent association–dissociation is not known, but Protein 4D or 4-1 is the largest one at pH 5.5, and it appears to give three sub-peaks on Sephadex G-200 chromatography, but the proteins of each of these sub-peaks behaved similarly on Phoroslide electrophoresis. However, if one uses the equation of Atassi and Gandhi\(^{19}\) of $s_{20} = 1.62 + (4.170 \times 10^{-9}) M$ to calculate the molecular weight of the $s_{20} = 6.25 S$ fraction, a mol. wt of about 110,000 is obtained, which is that calculated for Protein 4-2 by gel chromatography.

The data obtained indicate that Protein 4C obtained by electrophoresis and Protein 4-2 isolated by gel chromatography are the same protein and that this protein is the principal growth inhibitor of raw *P. vulgaris*. The growth inhibitor is a protein of mol. wt of 110,000 and isoelectric point of pH 5.2. The growth inhibitor is also a phytohemagglutinin with leucocyte stimulating activity, and from the molecular weight and amino acid content it appears to be similar to phytohemagglutinin pHA-a' of Dahlgren *et al*.\(^{20}\).

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REFERENCES


GROWTH INHIBITOR OF BEANS
16 Bowman, D. E. (1948) Arch. Biochem. 16, 109–113
19 Atassi, M. Z. and Gandhi, S. K. (1965) Naturwissenschaften 52, No. 10, 259