Isolation of a Trypsin Inhibitor from Navy Beans by Affinity Chromatography[1,2]

J. C. GOMES, U. KOCH, and J. R. BRUNNER, Department of Food Science and Human Nutrition, Michigan State University, East Lansing 48824

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ABSTRACT

A protein fraction that strongly inhibited the enzymatic activity of trypsin and chymotrypsin was separated from navy beans, Sanilac cultivar (*Phaseolus vulgaris* L.), by affinity chromatography with trypsin immobilized on agarose (Sepharose 4B). Under isoelectric focusing, the inhibitor revealed two zones at pH values of 4.40 and 4.45 for the major and minor zones, respectively. A molecular weight of 16,600 for the major component was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A molecular weight of 11,900 was estimated from an inhibitor-trypsin interaction. And, finally, a minimum weight of 12,214 was calculated from amino acid data, based on the limiting amino acid, methionine. Absence of tryptophan, low methionine, and high half-cystine contents characterized the inhibitor. Its composition is similar to an inhibitor isolated previously from navy beans by ion exchange on diethylaminoethyl cellulose and gel filtration.

Legume seeds have relatively high protein content and account for a significant proportion of the protein in the human diet. Unfortunately, legume seeds contain antinutritional factors that, if not inactivated, exert undesirable effects on their nutritive value (Puztai 1967, Jaffe 1975). Among these undesirable factors are the proteinase inhibitors.

Read and Haas (1938) were among the first to recognize trypsin inhibitor in plant material. Bowman (1944) described trypsin inhibitors in navy beans, soybeans, and corn. He suggested that a heat-labile protein in the aqueous extract of navy beans and soybeans, which inhibited the in vitro digestion of casein by trypsin, might account for the low nutritive value of raw legumes. Soybean and navy bean trypsin inhibitors were differentiated by Bowman (1947) on the basis of solubility and inhibitory activity.

Wagner and Riehm (1967) isolated an inhibitor from navy beans (California small white) by gel filtration and ion-exchange on diethylaminoethyl (DEAE) cellulose. Gel filtration data obtained with the oxidized protein indicated that the native protein exists as a single polypeptide chain. Ultracentrifugation characterization yielded a molecular weight of 23,000. Kinetic parameters of the reaction with trypsin yielded 11,500 mol wt. Bowman (1971) and Whitley and Bowman (1975) isolated from the seeds of navy beans (Sanilac) a trypsin inhibitor with 7,900 mol wt, as estimated from the inhibitor-trypsin interaction. From this value and its amino acid composition, they classified the inhibitor as one of the low molecular weight proteins known as Bowman-Birk inhibitors.

Nutritional implications of the trypsin inhibitor have been studied by Kakade and Evans (1965a, 1965b, 1966) and Kakade et al (1969b, 1970). The growth-inhibiting effect of raw navy beans has been attributed to toxic material other than the trypsin inhibitor and/or hemagglutinin (Kakade and Evans 1965a), a heat-labile growth-inhibiting agent as well as a methionine deficiency (Kakade and Evans 1965b), activity of intestinal microflora (Jayne-Williams 1977), and a disproportionately high concentration in the inhibitor of total cystine, which is resistant to enzymatic attack (Kakade et al 1969a).

The function of plant proteinase inhibitors has been a subject of speculation. Various proteinase inhibitors are easily extracted in active form, indicating that they are not bound to or associated with proteolytic enzymes. Some of these proteins inhibit the proteolytic enzymes of insects but rarely the proteolytic enzymes of plant origin (Birk 1968). Thus, proteinase inhibitors from plant origin have been regarded as specific metabolic defense agents against insects (Green and Ryan 1972, Steffens et al 1978).

MATERIALS AND METHODS

Navy beans, Sanilac cultivar (*Phaseolus vulgaris* L.), from the 1976 harvest were supplied by the Michigan State University Bean and Beet Research Farm (Saginaw) through the courtesy of Dr. M. W. Adams. Trypsin (bovine pancreas, twice crystallized, dialyzed, and lyophilized; salt free), soybean trypsin inhibitor (type I-S), and agarose (Sepharose 4 B-200 Pharmacia Fine Chemicals) were obtained from Sigma Chemical Co. Acrylamide and *N'N'* methylenebisacrylamide were obtained from the Ames Company and recrystallized from acetone before use. Ampholine, pH 4–6, 40% w/w, was from LKB. All other chemicals were reagent grade.

Immobilization of Trypsin on Agarose

Activated agarose was prepared as described by Cuatrecasas and Anfinsen (1971). Washed agarose (100 ml) was mixed with 100 ml of distilled water to which 20 g of finely ground cyanogen bromide was added. The reaction mixture was maintained at pH 11 and 20°C for 10 min, then transferred to a coarse sintered-glass funnel and washed under suction with 300–400 ml of cold $NaHCO_3$ (0.2*M*, pH 9.0) solution. Following a procedure adapted from Loeffler and Pierce (1973), the activated agarose was added to a cold solution of 11-amino undecanoate methyl ester (9 g in 150 ml of 0.2*M* $NaHCO_3$, pH 9.0), stirred overnight at 4–5°C, washed stepwise with water, 1*M* HCl, and water (to neutrality) and finally, with methanol. The synthesis of 11-amino undecanoate methyl ester was performed according to McKay et al (1958).

The agarose-coupled spacer was derivatized to its azide through a two-step protocol: First the gel volume was adjusted to 300 ml with methanol, 10 g of hydrazine was added, and the gel was stirred 6–7 hr at room temperature. After being washed with methanol and water to neutrality, the gel was made to 200 ml with water, acidified with 20 ml of 1*M* HCl, and treated with sodium nitrite (3 g of $NaNO_2$ in 10 ml of water). After being stirred for 20 min at 0–2°C, the derivatized gel was filtered, washed with water, and made to 200 g with borate buffer (0.1*M* $CaCl_2$, 0.001*M* HCl, 0.02*M* H_3BO_3; pH 4.1).

Trypsin was immobilized on the derivatized agarose as follows: A solution containing 2.5 g of trypsin in 100 ml of borate buffer, pH 4.1, was added to the gel suspension, which was adjusted to pH 9.0 with 5*N* NaOH and stirred for 20 hr at 0–2°C. The suspension was filtered and washed with small portions of borate buffer, pH 4.1, to remove free trypsin, which was quantitatively measured in the combined filtrate and washings.

The washed, gel-bound trypsin was suspended in 100 ml of buffer (0.1*M* NH_4Cl, 0.1*M* NH_4OH, 0.1*M* $CaCl_2$; pH 9.0), stirred for 4 hr at 0–2°C, filtered, washed, made up to 200 ml with the pH 3.8 solution (0.1*M* $CaCl_2$, 0.001*M* HCl), and stored at 4–5°C until used. Sodium azide was added as a preservative. The procedure is represented schematically in Fig. 1.

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Esterase activity of the immobilized trypsin was assessed with a pH-Stat (Sargeant Co.), employing *p*-tosyl-L-arginine methyl ester as the substrate (Walsh and Wilcox 1970).

Protein Extraction

Two-hundred grams of navy bean powder (50 mesh) was added to 1 L of 1*M* NaCl, homogenized with a Super Dispax (Tekman Co. Mod. SD 45K), adjusted to pH 7 with sodium hydroxide, and stirred overnight at 4–5° C. The suspension was centrifuged at 16,000 × *g* (max) in a Sorvall, RC2-B, for 30 min at 4–5° C. The precipitate was washed with 1*M* NaCl, recentrifuged, and discarded. The combined supernatants were centrifuged at 35,000 × *g* (max) for 1 hr at 4–5° C and the precipitate discarded. The supernatant was dialyzed for 4–5 days against several changes of distilled water. The coacervated globulin fraction was separated by centrifugation at 16,000 × *g* (max) for 2 hr at 4–5° C. The washed pellet was freeze-dried and stored at 4–5° C for subsequent experiments.

The albumin-rich supernatant was pervaporated to about one-half volume in cellulosic dialysis bags at 4–5° C, freeze-dried, and stored at 4–5° C.

Proteins were extracted from heat-treated beans (121° C, 30 min) by a similar procedure. No globulin fraction was encountered in this isolation.

Separation of the Trypsin Inhibitors

An affinity column was prepared by pouring 100 ml of the agarose-trypsin preparation into a 50 × 2.5 cm glass column (Pharmacia Fine Chemicals). The column was washed with 300 ml of buffer (0.2*M* KCl adjusted to pH 2.0 with HCl) and with two column volumes of Tris buffer (0.05*M* Tris, 0.10*M* KCl, 0.02*M* $CaCl_2$; pH 8.0) before the bean extract was applied.

Samples were prepared by dissolving the freeze-dried albumin fraction in Tris buffer, pH 8.0, to give 1% solutions, which were filtered through a 5-μm Millipore filter. Solution volumes ranging from 50 to 250 ml were percolated through the packed column at a flow rate of 2 ml/min, followed by a wash with Tris buffer, which was continued until absorbance of the eluate measured at 254 or 280 nm, was reduced to a low level. The eluting solvent was changed to pH 2.0 to enhance dissociation of the trypsin-inhibitor complex (Mosolov and Fedurkina 1974). After elution of the inhibitor (inhibitor peak: 20–50 ml), the column was washed with the $CaCl_2$ solution (0.1*M*, pH 3.8), containing 0.2% sodium azide, and stored at 4–5° C for reuse. The inhibitor fraction was dialyzed for 1–2 days against distilled water, freeze-dried, and stored at 4–5° C.

Inhibition Measurements

Trypsin activity in the presence of the affinity-isolated trypsin inhibitor or of other bean protein fractions was measured by a modification of Kunitz's method (1947), as employed by Kakade et al (1969b). The effective range of trypsin concentration and hydrolysis time was established from several enzyme concentrations and reaction times. For inhibition measurements, solutions of 50 μg/ml of navy bean trypsin inhibitor (NBTI), 500 μg/ml of globulin fraction, 200 μg/ml of albumin fraction, and 1 mg/ml of heat-treated navy bean protein (HTBP) were employed. All

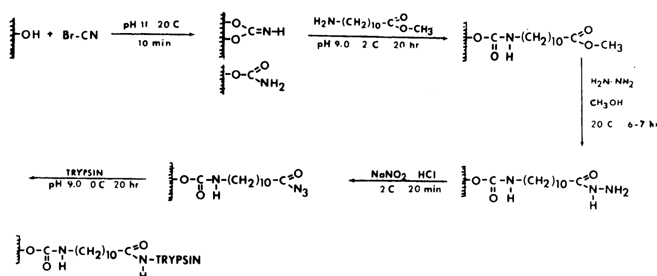

Fig. 1. Immobilization of trypsin on agarose. Adapted from Porath and Axen (1976) and Loeffler and Pierce (1973).

solutions were prepared in phosphate buffer (0.1*M*, pH 7.6). One milliliter of trypsin solution (50 μg/ml) was added to each tube and the mixture incubated for 10 min at 37° C. After incubation, 6.0 ml of 5% trichloroacetic acid (TCA) was added to one tube that served as a blank, for each level of protein examined. Then, 2.0 ml of 2% casein solution, previously adjusted to 37° C, was added to the experimental tubes. After 20 min the reaction was stopped by adding 6.0 ml of 5% TCA, and the tubes were allowed to stand for 1 hr at room temperature. After the precipitate was removed by filtration, the solution was monitored at 280 nm against its appropriate blank. Absorbances were transformed to $A^{3/2}$, as proposed by Miller and Johnson (1951), for calculating the residual activities.

Amino Acid Analyses

Tryptophan was determined colorimetrically after partial hydrolysis with pronase and reaction with dimethylaminobenzaldehyde (Spies 1967, Spies and Chambers 1948).

Amino acid were analyzed on 24 and 72-hr hydrolysates, employing a Beckman amino acid analyzer, Model 120 C, according to the procedures of Moore et al (1958). Cystine and methionine were determined by the methods of Schram et al (1954) and Lewis (1966), employing performic acid oxidation of methionine and cystine to methionine sulfone and cysteic acid, respectively.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gels were prepared and processed essentially as described by Weber and Osborn (1969). Four concentrations of acrylamide (5.0, 7.5, 10.8, and 12.5% total gel concentration; 2.6% cross links) were used to facilitate data reduction to Ferguson plots (Rodbard 1976). Samples ranging from 20 to 40 μl and containing 1 mg/ml of protein were layered on top of the gels. Electrophoresis was conducted at room temperature with a constant current of 8 mA per tube. Gels were soaked in 5% TCA for 30 min, stained with Coomassie Brilliant Blue R-250 for 1–2 hr and destained by diffusion in water/acetic acid/methanol (15:10:175).

Isoelectric Focusing

Isoelectric-focusing gel electrophoresis was performed according to the method of Wrigley (1971). NBTI samples were mixed with the acrylamide solution (7.8% total gel concentration), which contained ampholyte, pH 4–6, and riboflavin. This solution was transferred to gel tubes and exposed to a fluorescent light for polymerization. Electrophoresis was performed in a water-cooled cell at 0–2° C). A current of 2 mA per tube was applied to 400 V, then stabilized at this potential for 4–5 hr. The pH gradient was determined by cutting three gels into 15 pieces and was monitored with a microelectrode (Micro Electrodes, Inc.). The remaining gels were stained by Malik and Berrie's method (1972) or by the procedure employed for the SDS-PAGE gels.

RESULTS AND DISCUSSION

Immobilized Trypsin and Separation of Inhibitor

Agarose-bound trypsin was measured by calculating the difference between the initial amount added and that recovered in the washings. Esterase activity of the immobilized enzyme, determined by measurements with a pH-Stat, demonstrated that 17.4–19.5% of the activity of native trypsin was retained. There was no significant loss in activity during six months of storage under the conditions described. Agarose beads, both before and after activation and coupling, had previously been observed to exhibit very little nonspecific adsorption of proteins. This finding was verified by percolation of soybean trypsin inhibitor (SBTI) and ovalbumin on an experimental column (Cuatrecasas and Anfinsen 1971).

Immobilization of trypsin on porous glass showed nonspecific binding of proteins, as verified when casein and ovalbumin were percolated through a column according to the method of Loeffler and Pierce (1973).

Percolation of the albumin fraction through the agarose affinity column and elution with acidic solution yielded a preparation containing a strong inhibitor of trypsin and α-chymotrypsin. This fraction contained 60% protein (N × 6.25) and comprised 2.7% of the protein in the albumin fraction. An affinity chromatogram illustrating a separation of NBTI is shown in Fig. 2.

Inhibition Measurements

The action of NBTI, albumin, globulin, and HTBP on the hydrolysis of casein by trypsin is shown in Fig. 3. Globulins and HTBP had little or no effect on trypsin activity. NBTI strongly inhibited the hydrolysis of casein both by trypsin and by α-chymotrypsin. Similar observations regarding trypsin inhibitors in the water-soluble portion of bean protein have been reported (Bowman 1944, 1971; Kakade and Evans 1965a; Mosolov and Fedurkina 1974; Seidl et al 1969).

Amino Acid Composition

Amino acid composition of NBTIs are reported in Table I. Important aspects of these data are the absence of tryptophan, low content of methionine, and high content of half-cystine. Systematic trends in composition pointed out by Birk (1968) for other trypsin inhibitors from plant origin were verified with the NBTI: namely, unusually high and constant content of proline and of acidic and basic amino acids; high percentage of serine and threonine associated with high content of cystine; low content or absence of tryptophan; and the small amount of methionine.

Legume proteins have low biological values due to a major deficiency in availability of sulfur-containing amino acids. Any improvement in amino acid balance would significantly improve the nutritional quality of legumes (Padhye and Salunkhe 1977). The relatively high content of half-cystine in the NBTI, its lability to heat treatment, and the possibility that its inactivated form is readily digestible constitute reasons for proposing that high levels of the component in legumes may be a desirable characteristic.

Isoelectric Focusing

The principal and minor protein zones of the isoelectrically-focused NBTI fraction were located at pH values of 4.40 and 4.45, respectively. The zones were visible only after staining with Coomassie Blue R-250 because no precipitation occurred at their isoelectric pH as had been observed for SBTI.

Molecular Weight Determinations

The amount of inhibitor required to completely inhibit the activity of a given amount of enzyme can be determined by extrapolation of a plot of "remaining activity" vs "inhibitor added" to zero enzymatic activity. From these data, the inhibitor molecular weight and dissociation constant of the enzyme-inhibitor complex (Green and Work 1953) or the percentage of active enzyme (Wagner and Riehm 1967) can be estimated.

The activity of trypsin employed in this experiment was determined with SBTI. The method yielded a molecular weight of 12,000 for SBTI. Assuming a molecular weight of 21,500 for the SBTI (Wu and Sheraga 1962), we calculated that the trypsin employed for immobilization was 55% active. This correction factor was used when the molecular weight of the NBTI was determined.

Assuming a 1:1 molar ratio for the trypsin-NBTI complex, a molecular weight of 11,900 was obtained. The dissociation constant of the trypsin-NBTI complex, estimated from the residual trypsin activity at the equivalence point, was 7.6×10^{-10} *M* and was computed from one enzyme concentration only. The data plot is shown in Fig. 3A.

TABLE I
Amino Acid Composition of Navy Bean Trypsin Inhibitor

Amino Acid	Residues[a]	Mole (%)	Value[b]
Lysine	6	5.17	5.27
Histidine	5	5.04	4.95
Arginine	4	3.03	3.50
Tryptophan[c]	0	0.0	0.0
Aspartic acid	16	14.43	14.39
Threonine	7	6.32	6.49
Serine	18	16.02	16.64
Glutamic acid	10	9.29	8.30
Proline	8	7.10	7.79
Glycine	3	2.44	2.17
Alanine	4	3.77	3.72
Half cystine[d]	14	12.36	14.38
Valine	3	2.26	1.04
Methionine[d]	1	0.90	0.62
Isoleucine	5	4.83	4.33
Leucine	4	3.65	2.92
Tyrosine	2	1.75	1.70
Phenylalanine	2	1.90	1.79

[a] Based on methionine as limiting amino acid.
[b] Data from Wagner and Riehm (1967).
[c] Determined according to Spies (1967) and Spies and Chambers (1948).
[d] Determined as cysteic acid and methionine sulfone, respectively.

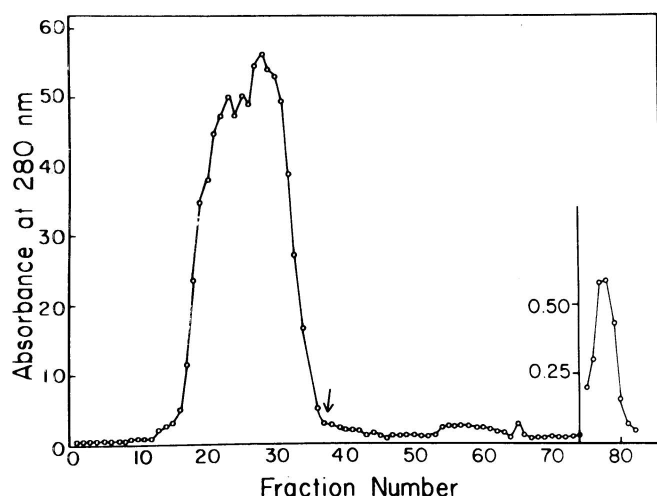

Fig. 2. Affinity separation of navy bean trypsin inhibitor. Fifty milligrams of the albumin fraction was added to a column (18.5 × 2.5 cm) of agarose-bound trypsin. Elution was at the rate of 2 ml/min. Arrow indicates change in elutant from Tris buffer pH 8.0 to 0.2*M* KCl, pH 2.0. Five-milliliter fractions were collected. The inhibitor is in fractions 76–82.

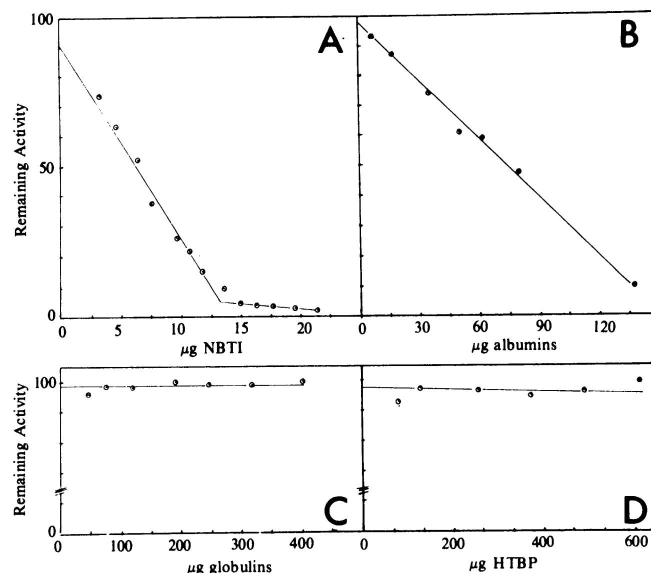

Fig. 3. Hydrolysis of casein by trypsin in relation to the level of: **A**, navy bean trypsin inhibitor; **B**, albumins; **C**, globulins; **D**, proteins extracted from heat-treated seeds.

A minimum molecular weight calculated from the amino acid composition, considering methionine as the limiting amino acid, was 12,214 (Table I). The average of the values calculated from alanine, glycine, phenylalanine, and glutamic acid residues was essentially similar.

The molecular weight estimated from SDS-PAGE gels was 16,600 for the major electrophoretic zone. Although the value obtained is informative, the results must be analyzed carefully. The basic assumption for the accuracy of molecular weight determinations on SDS gels is that SDS molecules bind to the protein with a constant ratio and that separation is due to size (Weber and Kuter 1971, Svasti and Parrijpan 1977). Abnormal binding of SDS by some proteins or a typical conformation of the protein-SDS complex can affect the mobility of proteins in SDS gels (Banker and Cotman 1972). The ideal situation, in which the presence of highly charged ionic detergent results in a uniform charge density for all proteins, including both standards and unknowns, is only rarely achieved in practice (Rodbard 1976). When SBTI was included in the set of standard proteins, the resulting mobility was much higher than expected for its reported molecular weight. The major band of NBTI had a mobility very close to that of SBTI. Thus, a molecular weight of about 21,000 may be more plausible than is the value reported above.

Ferguson plots of SDS-PAGE mobilities for NBTI and SBTI indicated that their free mobilities (*Yo*) were similar, 1.20 and 1.21, respectively. Four other proteins (bovine serum albumin, ovalbumin, α-chymotrypsinogen, and lyzozyme) demonstrated considerable systematic variations in *Yo*; ie, *Yo* increased with increasing molecular weight. Equality of *Yo* values and no systematic trend between *Yo* and molecular weight or between *Yo* and retardation coefficient (Kr) are necessary conditions for molecular weight estimations from PAGE data from a single gel concentration (Frank and Rodbard 1970).

When these conditions are not achieved, estimation of molecular weight from a relationship between molecular weight and Kr is preferable because it contains information from several gel concentrations (Rodbard 1976, Ugel et al 1971). A plot of molecular weight vs Kr for the calibration employed was linear. From this relationship, a molecular weight of 17,650 was derived for the NBTI.

The inhibitor isolated by Wagner and Riehm (1967) and by Whitley and Bowman (1975), as well as the specimen reported here, have similar amino acid profiles (Table I). Oxidation of disulfide bonds as performed by Wagner and Riehm (1967) does not eliminate the possibility of associations through other residues (Birk 1976). When dissociating agents such as a combination of SDS and β-mercaptoethanol are employed, association through disulfide bonds and hydrophobic interactions should be eliminated. Therefore, the patterns observed by SDS-PAGE are presumed to be manifestations of the monomeric species. The hypothesis that the inhibitor has a low molecular weight but is retarded in SDS-gels due to conjugated carbohydrate moieties (ie, higher apparent molecular weight) is untenable because NBTI contains only two moles of hexose per mole (Wagner and Riehm 1967). Thus, in view of the results we obtained with SDS-PAGE, the seemingly high molecular weight of 23,000 as reported by Wagner and Riehm (1967) may not be due to molecular interactions, as proposed by Whitley and Bowman (1975). Further investigations employing additional dissociating agents, as well as a systematic study of NBTI by SDS-PAGE, should be undertaken before accepting an electrophoretically-derived molecular weight.

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