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Isolation of Trypsin Inhibitor from Navy Beans (Phaseolus vulgaris L.) by Affinity Chromatography presented by

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ISOLATION OF TRYPSIN INHIBITOR FROM NAVY BEANS (*Phaseolus vulgaris* L.) BY AFFINITY CHROMATOGRAPHY

By

Jose Carlos Gomes

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ABSTRACT

ISOLATION OF TRYPSIN INHIBITOR FROM NAVY BEANS (*Phaseolus vulgaris* L.) BY AFFINITY CHROMATOGRAPHY

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Legume grains are relatively high in protein content. Common beans (*Phaseolus vulgaris* L.) are one of the major sources of protein in the world. Among undesirable nutritive factors in beans are the proteinase inhibitors.

A trypsin/chymotrypsin inhibitor present in the albumin fraction of navy beans _ sanilac cultivar - was separated by affinity chromatography on trypsin immobilized on agarose beads. Molecular weights calculated by SDS-PAG electrophoresis were 16,600 and 33,800 for the major and minor bands, respectively. Molecular weight estimated from inhibition measurements was 11,900; minimum molecular weight, based on methionine as limiting amino acid was 12,214. Dissociation constant of trypsin-inhibitor complex was 7.6×10^{-10}M. Isoelectric pH's were at 4.40 and 4.45 for the major and minor bands, respectively. Amino acid analysis showed that the inhibitor has a high content of half-cystine, a low amount of methionine and no tryptophan.

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INTRODUCTION

A significant proportion of the protein in the human diet, on a worldwide basis, comes from legumes. It is well known that common beans (Phaseolus vulgaris L.) are one of the main sources of protein in Latin America and particularly in Brazil where several dietary surveys have shown an average daily consumption of 60 g of beans per person (De Souza et al., 1973). Unfortunately they contain a great number of antinutritional factors which, if not destroyed, have undesirable effects on the nutritive value of these seeds. Raw beans when fed in an experimental diet are toxic to animals (Jaffe, 1972). Among these undesirable factors are the proteinase inhibitors.

Trypsin inhibitors from navy beans (Phaseolus vulgaris L.) have been prepared by fractional precipitation, gel filtration and ion-exhcange chromatography or combinations of these techniques.

Conventional procedures of protein separation and purification are generally based on small differences in physiochemical properties of proteins. Generally these techniques are tedious, yield poor resolution and selectivity. Affinity chromatography exploits the biological specificity of the protein-ligand interaction. In principle,

molecules with appreciable affinity for the ligand will be retained and others will pass through the column unretarded. The adsorbed proteins are eluted by altering the composition of the solvent to favor dissociation of the liquid-protein complex (Cuatrecasas and Anfinsen, 1971).

It was the purpose of this study to separate a trypsin inhibitor from navy beans on the basis of the specific binding with trypsin immobilized on an insoluble support.

LITERATURE REVIEW

Isolation and Characterization of Navy Bean Trypsin Inhibitors

Read and Haas (1928) were among the first workers to recognize the presence of a trypsin inhibitor in plant material. They reported that an aqueous extract of soybean flour inhibited the ability of trypsin to liquify gelatin. No attempt was made to separate the inhibitor.

Bowman (1944) described the presence of trypsin inhibitors in navy beans, soybeans, wheat and corn. He was the first to suggest that the presence of a heat labile protein in aqueous extracts of navy beans and soybeans, which inhibited *in vitro* digestion of casein by trypsin, might account for the low nutritive value of raw legumes. He reported that the digestion retarding fraction from navy beans could be concentrated by precipitation with acetone or alcohol while satisfactory precipitation from soybean extract could be achieved with acetone only.

Kunitz (1945, 1946) succeeded in crystallizing a trypsin inhibitor protein of globulin type from the extracts of soybean which later became the most extensively studied inhibitor of plant origin. Soybean and the navy bean trypsin inhibitors were differentiated by Bowman (1948) in regard to

their solubilities and activities. He found that the crude navy bean inhibitor was considerably more active and water soluble than the crystalline, globulin soybean trypsin inhibitor.

Additional studies were conducted on the trypsin inhibitor of navy beans. Wagner and Riehm (1967) isolated a trypsin inhibitor from navy beans (California small white bean) by first extracting the ethanol washed ground seeds with low concentration hydrochloric acid followed by ammonium sulfate fractionation, gel filtration and ion-exchange chromatography on DEAE-cellulose. Important aspects of its amino acid composition were the low level of methionine, the absence of tryptophan and the high content of half-cystine. The inhibitor contained 2 moles of hexose per mole of protein and no thiol groups. Gel filtration of the oxidized protein indicated that the native protein was a single polypeptide chain. Ultracentrifugation data gave a molecular weight of 23,000. The stoichiometry of the reaction between inhibitor and trypsin suggested an inhibitor to enzyme molar ratio of 1:2.

Bowman (1971) chromatographed the water extract of navy bean (Sanilac) on a DEAE-cellulose column with NaCl gradient. Except for the first peak eluted without the aid of NaCl, the entire chromatographic elution pattern represented inhibiting material. One of the components was further investigated. This component was homogeneous on disc electrophoresis and inhibited the proteolytic and

esterolytic activities of both trypsin and α-chymotrypsin. The constant apparent specific activity of this navy bean inhibitor in reaction with trypsin, containing varying proportions of active and inactive enzyme, suggested that the inhibitor reacts with active as well as inactive trypsin.

In a subsequent experiment, Whitley and Bowman (1975) investigated the water-extractable proteins of navy beans. The fraction containing inhibitors was eluted from a DEAE-cellulose column with 0.13 M NaCl. This fraction was rechromatographed on the same column using NaCl and pH gradients. Four components were separated of which one was shown to be homogeneous and one nearly so. The molecular weight for the homogeneous component calculated by reaction with trypsin was 7,900. Based on this value and in the content of characteristic amino acids in both components, the authors place them as members of a general class of low molecular weight proteins known as Bowman-Birk inhibitors. They explained that the possibility of self-association was the reason why Wagner and Riehm (1967) reported a much higher molecular weight for one inhibitor with an amino acid composition similar, on a relative basis, to the two components investigated.

It should be noted that the methods thus far described for calculation the molecular weight of the navy bean inhibitor did not take advantage of dissociating agents like a combination of sodium dodecyl sulfate (SDS) and

mercaptoethanol. Oxidation of dissulfide bonds as performed by Wagner and Riehm (1967) does not eliminate the possibility of association through other residues. Intermolecular association for the Bowman-Birk inhibitor from soybeans does not involve disulfide bonds (Birk, 1976).

Nutritional Importance

Early in this century Osborn and Mendel (1912) showed that phaseolin and raw navy bean meal were not capable of supporting animal growth. McCollum *et al.* (1917) fed navy bean meal as a source of protein and the animals failed to grow. These authors attribute the failure of navy beans to promote growth to the presence of hemicellulose. Johns and Finks (1920) supplemented the fraction phaseolin and navy bean meal with cystine after heat treatment to obtain normal growth. They suggested that the beneficial effect of heat alone could be destruction of toxic material or improvement in digestibility of the proteins. Waterman and Johns (1921) tested the later hypothesis. They found that heat treatment of the navy bean protein increased the *in vitro* digestion by trypsin and pepsin.

Everson and Heckert (1944) studied the biological value of several legume seeds, including navy beans. They reported appreciable differences in the growth-promoting qualities between raw and cooked beans.

Kakade and Evans (1965a) isolated five protein fractions from navy beans containing trypsin inhibitor and

hemagglutinin activity in different proportions. These fractions were combined with autoclaved beans, or with raw beans followed by autoclaving. All of those fractions were found to inhibit the growth of rats. The growth inhibitory effect was attributed neither to the hemagglutinin nor the trypsin inhibitor, but to a toxic material scattered throughout all navy bean fractions. In a subsequent experiment, Kakade and Evans (1965b) suggested that the low nutritive value of navy beans was due to the presence of a heat labile growth inhibitor and methionine deficiency. Autoclaving - 121°C; 5 min. - destroyed nearly all trypsin inhibitor and totally the hemagglutinin activity. Even when supplemented with the deficient amino acids, raw navy beans did not support the growth of experimental animals. This growth inhibitor factor was later isolated and characterized (Evans et al., 1973). It was identified as a phytohemagglutinin with leucocyte stimulating activity. Its molecular weight was 110,000 and possessed an isoelectric point at pH 5.2. This protein appears to be similar to the phytohemagglutinin pH A=a' (Dahlgren et al., 1970) in respect to molecular weight and amino acid composition.

Soaking the beans was found to decrease both trypsin inhibitor and hemaglutinin activity. Germination had the opposite effect on the inhibitor and did not change the hemagglutinin (Kakade and Evans, 1966).

Although comprising only 2.5% of the total protein, the trypsin inhibitor, present in a fraction isolated by

Kakade and Evans (1965a), contributes 40% of the total cystine of the navy beans. Since the navy bean trypsin inhibitor is poorly attached by digestive enzymes unless modified by heat, Kakade et al. (1969) suggested that this disproportionate distribution of cystine was the major factor involved in the low nutritive value of raw navy beans.

This resistance to enzymatic attack is probably due to the stability of molecule produced by a large number of disulfide bonds. The effect of heat is to cause an unfolding of the molecule resulting in the exposure of peptides bonds susceptible to enzymatic cleavage (Birk, 1968). In a related experiment Kakade et al. (1970) fed rats with a synthetic inhibitor - p-aminobenzalmidine - and navy bean trypsin inhibitor. The effects of antibiotic and cystine supplementation were investigated. Both inhibitors caused growth retardation, pancreatic enlargement, and exaggerated secretion of pancreatic enzymes. Cystine supplementation failed to bring the growth to the same level obtained with antibiotics. They explained the results on the basis of conversion of methionine to cystine to meet the demand created by abnormal secretion of pancreatic enzymes, and the increase in intestinal absorption of cystine promoted by antibiotics.

The existence of trypsin inhibitors in legume seeds seemed to offer a reasonable explanation for the observation made by the early investigators that heat treatment improved the nutritional value of these seeds. From the results of a

study on the physiological response of rats to raw or treated soybean meal diets, it was concluded that the trypsin inhibitors are responsible for the pancreatic hypertrophy and from 30 to 50% of the growth-inhibiting effect (Steiner and Frattali, 1969). In a review, Birk (1968) affirmed the pancreatic hypertrophic effect, but concluded that these inhibitors have a minor role in growth depression.

For a more comprehensive review of trypsin inhibitors of plant origin, the reader is referred to the reviews by Pusztai (1967) and Birk (1968). Distribution, occurrence, chemistry, structure and preparation of several plant proteinase inhibitors were covered by Birk (1976).

Significance in Plants

The function of plant proteinase inhibitors has been a subject of speculation. Various proteinase inhibitors are easily extracted in active form from their source, which indicates that they are not bound to or associated with proteolytic enzymes. Some of these proteins inhibit proteolytic enzymes of insects, but rarely proteolytic enzymes of plant origin. The arguments regarding plant proteinase inhibitors as specific metabolic defense mechanism agents against insects are based on their ability to inhibit insect digestive proteinases. This hypothesis is further supported by the finding that wounding of the leaves of potato or tomato plants by insects or by mechanical means induces an accumulation of proteinase inhibitors in both damaged and

adjacent leaves (Applebaum et al., 1964; Birk, 1968; Green and Ryan, 1972; Birk, 1976 and Steffans et al., 1978).

EXPERIMENTAL

Chemicals and Materials

Navy beans, Sanilac cultivar (P. vulgaris), obtained from the 1976 harvest, were supplied by the Bean and Beet Research farm - Saginaw. The principal chemicals used in this research are listed here: 11-aminoundecanoic acid which was used to synthetize the 11-aminoundecanoate methyl ester employed as spacer in the immobilization of trypsin, was obtained from Pfaltz and Bauer Inc. Amino propyl porous glass, containing 0.053 mmole of amino group/g of material, mean pore diameter of 518A^{o}, surface area of 60.2 m^2/g, 80-120 mesh, was purchased from Electro-nucleonics, Inc. Cyanogen bromide and hydrazine (anhydrous) were acquired from Pierce. Sepharose 4B-200, trypsin (from bovine pancreas, 2x crystallized, dialysed and lypophilized, salt free), α-chymotrypsin (from bovine pancreas, 3x crystallized, lyophilized, salt free), soybean trypsin inhibitor (type I-S), p-tosyl-L-arginine methyl ester (TAME), tris-hydroxymethyl amino methane (Trizma Base) and coomassie brilliant blue R-250 were obtained from Sigma.

Acrylamide and N'N' methylenebisacrylamide were obtained from the Ames Company and recrystallized from acetone before use. The N,N,N'N' tetramethyl ethylenediamine

and riboflavin were purchased from Eastman Organic Chemicals. Reagent grade ammonium persulfate was purchased from Baker Chemical Company. Ampholine pH 4-6, 40% w/w was obtained from LBK.

All other chemicals were of reagent grade.

Apparatus and Equipment

pH values were monitored with an Instrument Laboratory Inc., Model 245 pH meter or a digital, Chemtrix Model 60A equipped with glass electrodes. For most laboratory weighings, a top loading, direct reading Mettler type K-7 balance was used. Analytical weighings were performed on a Sartorius analytical single pan balance. Centrifugation was performed in a sorvall model RC2-B centrifuge using either the large capacity model GSA or the superspeed SS-34 rotors. A recording pH stat manufactured by E.H. Sargent and Company was used for activity measurements of immobilized trypsin on agarose.

The immobilized trypsin on agarose (Sepharose 4B) was packed in a Pharmacia 50 x 2.5 cm glass column. The eluates were monitored at 254 nm with a recording ultraviolet analyser, model US-2 and in some cases fractions were collected and absorbances measured at 280 nm. The U.V. monitor, the recorder and a fraction collector model 1100 were manufactured by Instrumentation Specialities Company. A Beckman DK-2A spectrophotometer equipped with silica cells - 1 cm path length - was used for all spectrophotometric

measurements.

An electrophoresis apparatus manufactured by Buchler Instruments and a Bi-Rad Laboratories model 400 power supply (voltage range 0-500V, and current 0-100 mA) were used for all SDS gel electrophoresis and isoelectric focusing runs. The excess dye was removed from the electrophoretic gels by means of a Bio Rad Model 170 diffusion destainer.

Protein solutions were freeze-dried on a laboratory-constructed lyophilizer. A micro-Kjeldahl apparatus was used for nitrogen determination. Amino acid analysis was performed on a Beckman Model 120C Amino Acid Analyser.

Methods

Synthesis of 11-aminoundecanoate Methyl Ester

Methylation of 11-aminoundecanoic acid was performed as described by McKay *et al*. (1958):50.0 g of 11-amino undecanoic acid was added to 1250 ml of 3.5 N hydrochloric acid in methanol. Crystallization of derivative was induced by allowing this solution to stand at room temperature for 16-20 hours. After one third of the methanol was removed in vacuum the remaining mixture was cooled ($4^{o}C$) and the crystals formed were recovered by filtration on Whatman No. 1 paper and air dried at room temperature.

Immobilization of Trypsin on Porous Glass

Immobilized trypsin on glass was prepared by the technique described by Loeffler and Pierce (1973): 1.5 g of amino propyl porous glass was suspended in 20 ml of distilled water and 2.0 ml of 4.0 M KCl was added. pH was adjusted to 10.0 with 5 N NaOH. With continued stirring at room temperature under a hood, a solution of 3.0 g of cyanogen bromide in 3 ml of tetrahydrofuran was added. 5 N NaOH was added dropwise to maintain the pH at 10.0 for about 10 min. The suspended glass was filtered and washed thoroughly with 0.1 M sodium bicarbonate at pH 9.0.

The "activated" glass was added to a cold solution of 11-amino undecanoate methyl ester - 3.90 g in 50 ml of

0.1 M sodium bicarbonate (pH = 9.0) and 50 ml of methanol. The suspension was adjusted to pH 9.0 and stirred at 4-5°C for 20 h. Following this treatment it was filtered on a coarse sintered glass funnel and washed stepwise with methanol, 0.1 M HCl and, finally, water.

Forty milliliters of methanol and 3 ml of hydrazine were added to the treated beads, stirred for 3 h at 4-5°C, filtered on coarse sintered glass, washed stepwise with methanol, 0.1 M hydrochloric acid and water to neutrality and, finally, with methanol. The product was air dried before continuing the next step in the derivatization. The glass-hydrazide derivative was suspended in 5.0 ml of 0.1 M hydrochloric acid. With continuous stirring, a solution of 1.50 g of sodium nitrite in 5.0 ml of water was added and reaction continued for 2 min at 0°C. The product was washed with cold water and 6.0 ml of a pH 4.1 buffer (0.1 M $CaCl_2$, 0.001 M HCl, 0.02 M H_3BO_3) was added.

The glass-azide derivative obtained by this procedure was suspended in a solution of 5.0 ml of buffer, pH = 4.1, containing 300 mg of trypsin. The system was adjusted to pH 9.0 with 5 N NaOH and stirred for 20 h at 0°C. The pH was checked twice and maintained at pH 9.0 during this operation. The reaction mixture was filtered on coarse, sintered-glass and washed with small amount of buffer pH = 4.1. Five milliliters of buffer pH 9.0 (0.1 M NH_4Cl, 0.1 M NH_4OH, 0.1 M $CaCl_2$) was added to the washed glass-trypsin derivative and stirred at 0°C for 4 h. The

suspension was filtered and washed with a solution of pH = 3.8 (0.1 M $CaCl_2$, 0.001 M HCl) at 0°C. The product was stored in this medium at 4-5°C with sodium azide added as a preservative against microbial growth. The immobilization steps described above are represented schematically in Fig. 1.

Immobilization of Trypsin on Agarose

Activated agarose was obtained by the method of Cuatrecasas and Anfinsen (1971). An experimental preparation using 20 ml of agarose-Sepharose 4B- was performed before the preparative immobilization.

One hundred milliliters of decanted agarose was mixed with 100 ml of distilled water. Twenty grams of finely divided cyanogen bromide was added in one addition to the stirred suspension. Immediately, the reaction was raised to pH 11 with 5 N NaOH. The temperature was maintained at about 20°C by adding pieces of ice as needed. The reaction was completed in 10 min as indicated by the cessation of base uptake. A large amount of ice was added to the suspension which was transferred to a coarse sintered-glass funnel and washed under suction with 300-400 ml of a cold solution of $NaHCO_3$ (0.1 M, pH 9.0).

Following a procedure described by Loeffler and Pierce (1973), the activated agarose was added to a cold solution of 9 g of 11-amino undecanoate methyl ester in 150 ml of 0.1 M $NaHCO_3$ pH = 9.0, the final volume adjusted to 250 ml

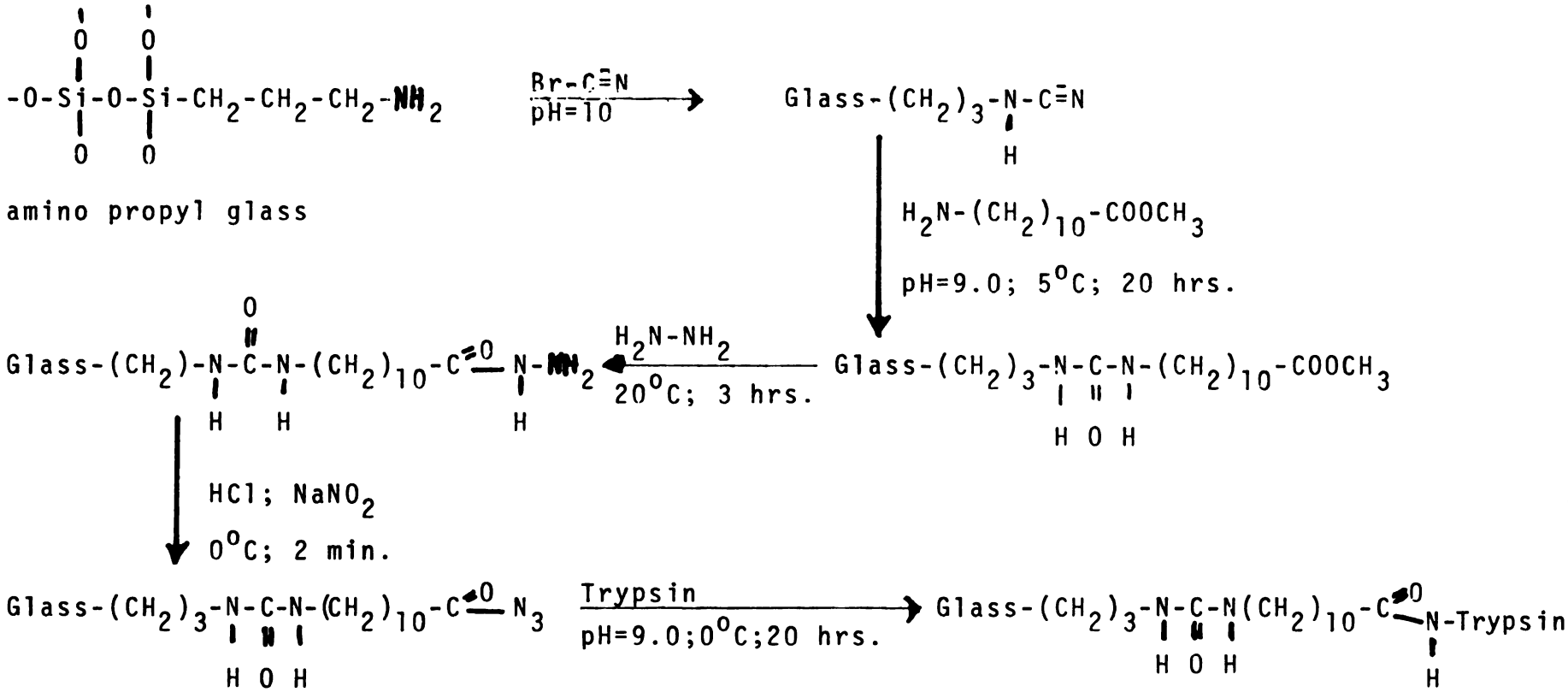


Figure 1. Mechanisms involved in the immobilization of trypsin on porous glass via the azide intermediate of 11-amino undecanoic acid (Weetall, 1975; Loeffler and Pierce, 1973).

with the same solution of $NaHCO_3$ and stirred overnight at 0-2°C. The product was filtered on a coarse sintered-glass funnel, washed stepwise with water, 1 M HCl and water to neutrality.

The activated gel was washed with six 50 ml portions of methanol, adjusted to 300 ml with methanol, and 10.0 g of hydrazine added. The suspension was stirred for 6-7 h at room temperature. The hydrazide derivative was filtered on a coarse sintered-glass funnel, washed thoroughly with methanol and water to neutrality, and finally adjusted to 200 g with water.

Twenty milliliters of 1.0 M HCl at 0°C was added to the cold suspension followed by 3.0 g of $NaNO_2$ dissolved in 10 ml of water. After stirring for 20 min at 0-2°C, the gel was filtered, washed thoroughly with water and then made to 200 g with buffer, pH 4.0 (0.1 M $CaCl_2$, 0.001 M HCl, 0.02 M H_3BO_3). Two and half grams of trypsin dissolved in 100 ml of buffer pH = 4.1 were added and pH adjusted to 9.0 with 5 N NaOH. The suspension was stirred at 9°C in a beaker covered with plastic film for 20 h. The pH was checked twice during this operation.

The reaction suspension was filtered and washed with small portions of buffer at pH = 4.1. Filtrate and washings were collected, volume adjusted to 500 ml (Filtrate I) with water and absorbance read at 280 nm. To the washed gel, 100 ml of buffer pH = 9.0 (0.1 M NH_4Cl, 0.1 M HN_4OH, 0.1 M $CaCl_2$) was added and the suspension stirred at 0°C for 4 h.

The suspension was filtered and washed with a cold solution at pH = 3.8 (0.1 M $CaCl_2$, 0.001 M HCl). As before, filtrate and washings were collected for absorbance readings (Filtrate II). Washed gel was made up to 200 ml with the same solution (pH = 3.8) and stored at 4-5°C with sodium azide added. The immobilization procedure is schematically represented in Fig. 2.

Determination of Unbound Trypsin

Absorbances of trypsin solutions ranging from 0.05 $mg.ml^{-1}$ to 0.5 $mg.ml^{-1}$ in buffer at pH = 4.0 were read at 280 nm - 1 cm - with the buffer serving as reference. Amount of unbound trypsin was estimated by reading the absorbance of filtrates and washings after addition of trypsin in the immobilization procedure.

Activity Measurements of Agarose-trypsin

Measurements of esterase activity of agarose-trypsin were performed using p-tosyl-L-arginine methyl ester as substrate as indicated by Walsh and Wilcox (1970).

Seven milliliters of 0.01 M p-tosyl L-arginine methyl ester (TAME) in buffer at pH = 7.95 (0.1 M KCl, 0.05 M $CaCl_2$, 0.01 M tris) was transferred to the sample cell of a Sargent pH-Stat. When the temperature of solution reached 37 ± 0.5°C, with the pH selector set at 8.0, the function dial was turned to "RUN". After the base line was stable, volumes ranging from 0.050 to 0.100 ml of agarose-trypsin

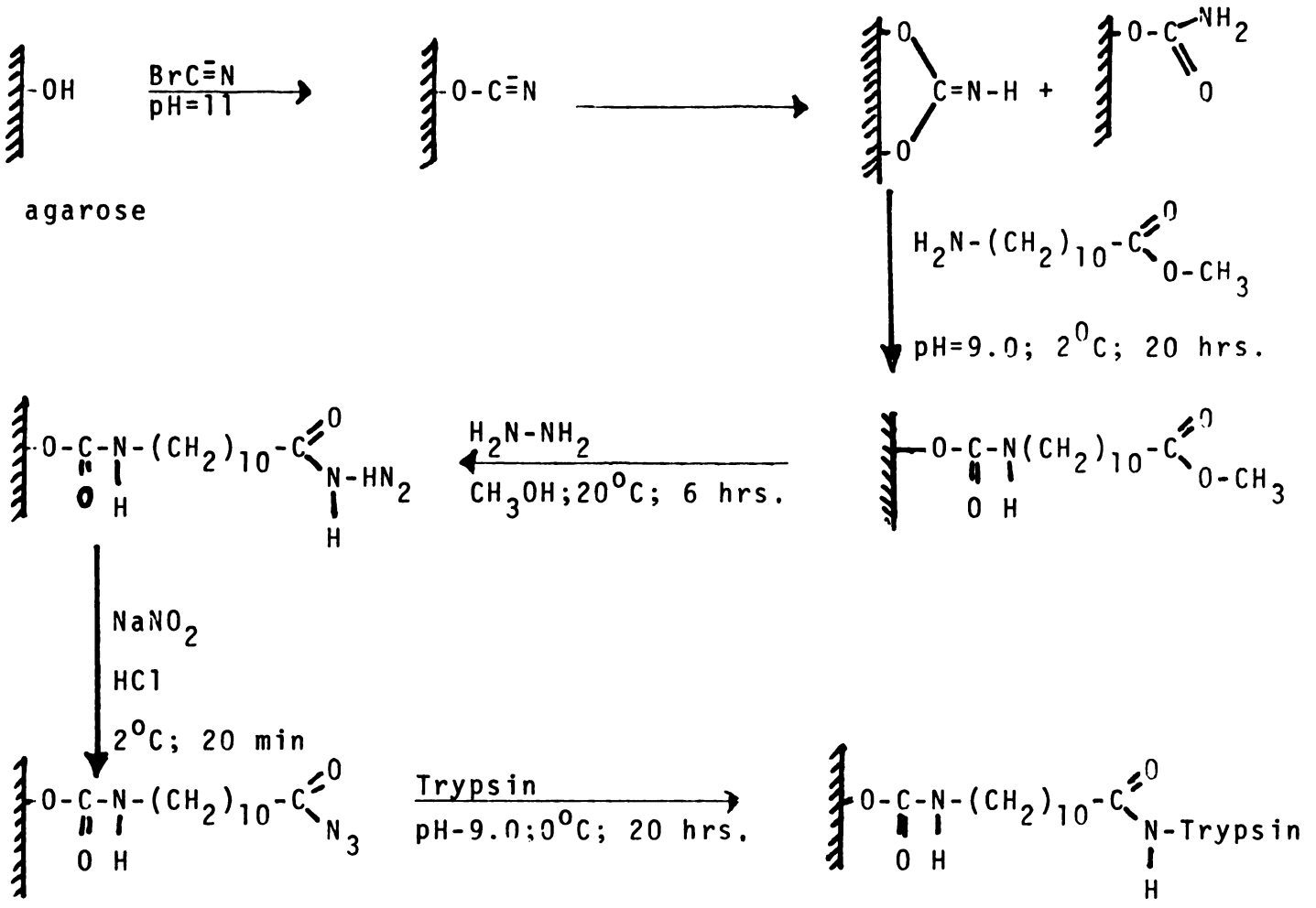


Figure 2. Immobilization of trypsin on agarose (Porath & Axen, 1976; Loeffler & Pierce, 1973.

suspension or from 0.020 to 0.050 ml of 0.1% trypsin solution in 0.001 M HCl were added with continuous stirring. During the first 3-5 min, the volume of 0.1 M tris base necessary to maintain the pH at 8.0 was recorded, and the initial slopes were determined for use in enzymatic activity calculations (Jacobsen et al., 1957).

Protein Extraction

Raw beans. The protein extraction procedure was adapted partially from Danielson (1950), Seidl et al. (1969), and Goa and Strid (1959). Two hundred grams of Navy bean (Sanilac) powder (50 mesh) was added to 1 liter of 1 M NaCl, homogenized with a Super Dispax (Tekmar Co. Mod. SD45K), adjusted to pH 7 with sodium hydroxide and stirred overnight at 4-5°C. The suspension was centrifuged at 16,000 x g for 30 min at 4-5°C. The precipitate was washed with 1 M NaCl, centrifuged again and discarded. The supernatant was centrifuged at 35,000 x g for 1 h at 4-5°C and the resulting precipitate was discarded. The classified supernatant was dialysed for 4-5 days against distilled water with periodic changes of water. The precipitated globulins were separated by centrifugation at 16,000 x g for 2 h at 4-5°C, washed with distilled water and centrifuged. The globulin fraction was freeze-dried and stored at 4-5°C.

The supernatant containing the albumins was pervaporated in dialysis bags overnight at 4-5°C until the volume

was reduced to about half of the initial volume, freeze-dried and stored at 4-5°C.

Heat treated beans. Before extracting the protein, 500 g of navy beans were treated at 121°C for 30 min. The cooked beans were air dried at 40°C overnight and ground in a Wiley mill to pass 50 mesh screen. Two hundred grams of this powder were treated similarly to the raw beans. There was no water-insoluble fraction in the 1 M NaCl extract.

Nitrogen Determination

Duplicate samples ranging from 4 to 15 mg of dried protein or about 100 mg of the 50 mesh powder were digested with 4 ml of the digestion mixture over a gas flame for 1 h. The digestion mixture consisted of 5.0 g of $CuSO_4 \cdot 5\ H_2O$ and 5.0 g of SeO_2 in 500 ml of concentrated sulfuric acid. After cooling the digestion mixture, 1 ml of 30% H_2O_2 was added to each flask and digestion continued for another hour. After cooling, the sides of the digestion flask were rinsed with small volumes of distilled water. The digestion flasks were connected to the distillation apparatus. Then, following neutralization with 25 ml of 40% sodium hydroxide solution, the released ammonia was steam distilled into 15 ml of 4% boric acid solution containing five drops of indicator consisting of 400 mg of bromocresol green and 40 mg of methyl red in 100 ml of 95% ethanol. Distillation was continued until a final volume of 50 to 50 ml of distillate was collected. The resulting ammonium borate was titrated

with 0.0193 N HCl. Hydrochloric acid was standardized with tris-hydroxymethyl amino methane (Sigma-Trizma base). Titrations were performed with a 10 ml burette. Recoveries of tryptophan standards performed in each run varied from 93.1 to 101.0% with an average of 97.9%.

Moisture Determination

The procedure consisted in weighing accurately, to the nearest 0.1 mg, a 4-5 g sample of 50 mesh navy bean powder into a previously dried and tared weighing bottle. The samples were dried at 70°C for 24 h. in a vacuum oven. At the end of drying time the vacuum was released with sulfuric acid dried air. The samples were cooled to room temperature in a desiccator and reweighed. Weight loss was assumed to be water.

Inhibition Measurements

Inhibition measurement with trypsin. Activity measurements of trypsin in the presence of inhibitor isolated from navy beans or other proteic fractions were performed by a modification of the method employed by Kunitz (1947) as proposed by Kakade et al. (1969).

In order to check the effective range of enzyme concentrations, 0.2 to 1.0 ml of 50 µg/ml of trypsin solution in 0.001 M HCl was pipetted into a triplicate set of test tubes - one set for each level of trypsin - and the final volume was adjusted to 2.0 ml with 0.1 M phosphate buffer

at a pH of 7.6. To one of the triplicate tubes (blank), 6.0 ml of 5% trichloroacetic acid was added and the tubes were set in a water bath at 37°C. Then 2.0 ml of a 2% casein solution, previously adjusted to 37°C, was added to each tube. The casein solution consisted of 2 g of casein dissolved in the phosphate buffer by heating on a steam bath for 15 min and made up to 100 ml. After exactly 20 min, the reaction was stopped by adding 6.0 ml of 5% TCA to the sample tubes. After standing for 1 h at room temperature, the suspension was filtered through Whatman No. 1, and absorbance of the filtrate was monitored at 280 nm against the blank for each level of enzyme. Hydrolysis time was checked with 1.0 ml of trypsin solution in intervals varying from 2 to 25 min of reaction. All conditions were the same as described for measurement of the effective range of concentrations.

For inhibition measurements, solutions of 50 µg/ml of navy bean trypsin in hibitor (NBTI), 50 µg/ml of soybean trypsin inhibitor (SBTI), 500 µg/ml of globulin fraction, 200 µg/ml of albumin fraction and 1000 µg/ml of heat treated navy bean protein were employed. All solutions were prepared in phosphate buffer.

Volumes from 0.0 to 1.0 ml of protein solution were pipetted into a triplicate set of tubes and adjusted to 1.0 ml of final volume with phosphate buffer. One milliliter of a 50 µg/ml of trypsin solution was added to each tube; the tubes were placed in a water bath at 37°C for

10 min. Finally, 6.0 ml of 5% TCA were added to one of the triplicate tubes which served as a blank for each level of protein examined. The remainder of the procedure was the same as that described above. In all cases, absorbances were transformed to $A^{3/2}$ as originally proposed by Miller and Johnson (1951).

Inhibition measurements with α-chymotrypsin. The procedure for determination of the activity of α-chymotrypsin in the presence of navy bean trypsin inhibitor (NBTI) was adapted from the method described by Rick (1974). Volumes from 0.0 to 1.0 ml of NBTI solution in the borate buffer were pipetted into a triplicate set of tubes and the volumes adjusted to 1.0 ml with the borate buffer. The borate buffer was prepared by addition of 6.06 g of H_3BO_3 and 50 ml of 1 N NaOH in 650 ml of distilled water, pH adjusted to 8.0 with hydrochloric acid and the volume completed to 1 l.

One milliliter of solution containing 48 μg/ml of α-chymotrypsin in 0.001 M HCl was added to each tube which were incubated at 35°C for 10 min. Then, 6.0 ml of 5% TCA was added to one of each triplicated set of tubes which served as blank. Two milliliters of casein solution, previously adjusted to 35°C, was added to each tube. The casein solution was prepared by suspending 2.0 g of casein in 95 ml of the borate buffer and heated on a steam bath for 10 min. One and one tenth milliliters of a 5% $CaCl_2$ solution was added and the final volume made to 100 ml after

cooling at room temperature. After 20 min, hydrolysis was stopped by the addition of 6.0 ml of a 5% TCA solution to the experimental tubes. After standing for 1 h at room temperature, the hydrolysis mixture was filtered and the absorbance of the TCA-soluble products was measured at 280 nm against the blank for each level of inhibitor.

Preparation of Affinity Columns

Experimental glass-trypsin column. One gram of glass-trypsin was mixed with 1.5 g of amino propyl glass and placed into a glass laboratory-made column. The packed column measured 3.50 x 1.25 cm. The column was washed with 1 M acetic acid, followed by tris buffer, pH 8.0, composed of 0.05 M tris-hydroxyimethyl amino methane, 0.10 M potassium chloride, 0.02 M calcium chloride and adjusted to pH 8.0 with hydrochloric acid. Solutions of soybean trypsin inhibitor (5 $mg.ml^{-1}$) were added to the column in two 1 ml portions. Protein solutions were prepared in tris buffer, pH 8.0. The charged column was washed with tris buffer, pH 8.0, until the absorbance (254 nm) was reduced to a low level. At this point the solvent was changed to 1 M acetic acid to elute the bound protein.

Experimental agarose-trypsin column. A glass column was packed with agarose-trypsin (4.5 x 1.25 cm) representing 5.0 ml of original Sepharose-4B. The column was washed successively with 1 M acetic acid and tris buffer, pH 8.0 (Filtrate III). Seven portions of 1.0 ml each of soybean

trypsin inhibitor and two of ovalbumin were added to the washed column. Both proteins were in a concentration of 1 $mg.ml^{-1}$. The remainder of the procedure was the same as described for the glass-trypsin column.

Preparative column. The preparative affinity-column was prepared by pouring agarose-bound trypsin, obtained from the initial 100 ml of agarose, into a 50 x 2.5 cm glass column. A sample applicator basket was placed on top of the column and the column was washed with about 300 ml of buffer, pH 2.0. The buffer consisted of a 0.2 M KCl solution which was adjusted to pH 2.0 with hydrochloric acid. Finally, the column was washed with 2 column volumes of tris buffer, pH 8.0, before applying the sample.

Separation of the Navy Bean Trypsin Inhibitor

Albumin solutions were prepared by dissolving the freeze-dried albumin fraction in the tris buffer, pH 8.0, to give 1% solutions. These solutions were filtered through a 5 μ-Millipore filter before applying to the affinity column.

Following a procedure employed by Mitchell et al. (1976), volumes of from 50 to 250 ml of the 1% albumin solution were percolated through the agarose-trypsin column at a flow rate of 2 ml/min. The column was washed with the tris buffer, pH 8.0. Washing was continued until absorbance of the eluate measured at 254 or 280 nm was reduced to a low level, thus eliminating non-inhibitor proteins. The

protocol for eluting the inhibitor was adapted from Mosolov and Fedurkina (1974). The eluting solvent was changed to a pH 2.0 buffer to enhance the dissociation of the trypsin-inhibitor complex. The inhibitor was eluted in a volume ranging from 20 to 50 ml. After the inhibitor peak was eluted, the column was washed with a pH 3,8 solution, containing sodium azide as a preservative against microbial growth. The column was stored at 4-5^{o}C and reused for 10 preparations of inhibitor.

The collected inhibitor fraction was dialysed for 1-2 days against distilled water, freeze dried and stored at 4-5^{o}C.

SDS-polyacrylamide Gel Electrophoresis

Polyacrylamide, sodium dodecyl sulfate (PAG-SDS) gels were prepared according to the method of Weber and Osborn (1969) with two modifications.

1. The proteins were incubated at 100^{o}C for 5 min. in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% SDS and 1% β-mercaptoethanol.
2. Four concentrations of acrylamide were used to facilitate data reduction to Ferguson plots.

Acrylamide and N,N' methylene bisacrylamide were recrystallized from acetone before preparation of gel solutions. All proteins were prepared in a concentration of 1 mg/ml except chymotrypsinogen and the navy bean trypsin inhibitor which were adjusted to 2 mg/ml. The density of

the samples was increased with sucrose and about 5 µl of bromophenol blue/50 µl of solution was added as tracking dye. 20 to 40 µl of sample was layered over the gels.

Electrophoresis was carried out at room temperature with a constant current of 8 mA/tube until the tracking dye migrated to within 5 mm of the lower end of the gel. The lower electrode served as the anode. Gels were soaked in 5% trichloroacetic acid for 30 min followed by staining with Coomassie Bri-liant Blue R-250 for 1-2 h at room temperature. Gels were destained by diffusion (circulation) in acetic acid-methanol-water (15:10:175).

Isoelectric Focusing

Isoelectric focusing gel electrophoresis was performed according to the method described by Wrigley (1971). The gels were prepared by mixing 7.70 ml of distilled water and 0.30 ml of navy bean trypsin inhibitor solution with 0.30 ml of carrier ampholyte solution - pH 4-6, 40% w/w - and 3.0 ml of acrylamide solution. After addition of 0.70 ml of 0.015% aqueous solution of riboflavin, this mixture was transferred to 75 x 5 mm glass tubes. Water was layered on top and the tubes were exposed to a fluorescent light for polymerization. The acrylamide solution consisted of 30 g of acrylamide and 1.2 g of N,N' methylene bisacrylamide, both cyrstallized from acetone and dissolved in water to a volume of 100 ml.

The tubes were placed in a Buchler disc electrophoresis apparatus cooled with water at 0°C. Sulfuric acid (0.2%) was used as the anodic solution and 0.4% ethanolamine as the cathodic solution. A current of 2 mA per tube was applied until the voltage required to yield this current increased to 400 v. The voltage was then stabilized at this level for 4-5 h. After focusing, the pH gradient was determined. Two or three gels were cut in 2.4 mm pieces which were macerated in 0.5 ml of freshly boiled and cooled water. The pH of the water extract was measured and plotted vs. length of the gel. Comparison gels were stained by the method of Malik and Berrie (1972) or by the procedure employed in SDS-PAGE.

Amino Acid Analysis

Tryptophan determination. Tryptophan was determined colorimetrically after a partial hydrolysis with pronase as described by Spies (1967) in procedure W. A 3.0 mg sample was weighed into a small glass vial fitted with a screw cap. To each vial, 100 µl of pronase solution was added. The pronase hydrolytic solution was prepared by adding 100 mg of pronase to 10 ml of 0.1 M phosphate buffer, pH 7.5. The suspension was shaken and clarified by centrifugation.

The vials were closed and incubated for 24 h at 40°C. Following incubation the vials were opened and 0.9 ml of phosphate buffer was added. These uncapped vials were

placed into Erlenmeyer flasks containing 9.0 ml of 21.2 N sulfuric acid and 30 mg of dimethylaminobenzaldehyde and their contents mixed by rotating the flasks. The reaction mixture was kept in the dark at room temperature for 6 h. Following the addition of 0.1 ml of 0.045% sodium nitrite solution, the reaction mixture was shaken and the color was allowed to develop for 30 min in the dark at room temperature. Absorbances were measured at 590 nm. Duplicate blanks of the pronase solution were run similarly, and the tryptophan content of pronase was subtracted from the total tryptophan contents. A standard curve from zero to 120 µg of tryptophan was prepared according to Spies and Chambers (1948). Absorbance and tryptophan levels were related by linear regression.

Amino acid composition. Amino acid analyses were performed on 24 and 72 h acid hydrolysates employing a Beckman Amino Acid Analyser, Model 120^{o}C according to the procedures of Moore et al. (1958). Eight milligrams of navy bean trypsin inhibitor was weighed into 10 ml glass ampoules and 5 ml of 6 N HCl was added to each of the ampoules. The content of the ampoules was frozen in dry ice-ethanol bath, evacuated with the lyophilizer vacuum pump, and allowed to melt slowly under vacuum to remove dissolved gases. The content was again frozen and sealed with an air-propane flame. The sealed ampoules were placed in an oil bath at 110^{o}C for 24 and 72 h.

The ampoules were broken on top and 1 ml of norleucine solution containing 2.5 μmoles/ml was added to each as a standard for transfer losses. The content of each ampoule was quantitatively transferred to an evaporating flask. The hydrochloric acid was removed on a rotatory evaporator. The dried sample was washed with a small amount of deionized water and again taken to dryness. In all, three washings were performed to remove residual HCl. The acid-free hydrolysate of each ampoule was transferred to 5 ml volumetric flask with citrate-HCl buffer, pH 2.2. An aliquot of 0.2 ml was applied to the analyser column. The chromatograms were quantitated by peak integration using a Spectra Physics Autolab System AA.

Methionine and cystine analyses. The methods of Schram et al. (1954) and Lewis (1966) were used. These methods involve performic acid oxidation of methionine and cystine to methioine sulfone and cysteic acid, respectively. Ten milligrams of navy bean trypsin inhibitor was weighed into 25 ml pear-shaped flask. The protein was oxidized for 15 h with 10 ml of performic acid at $4^{o}C$. After oxidation, 1 ml of norleucine (2.5 μmoles/ml) was added and the performic acid removed in a rotatory evaporator. The dried sample was quantitatively transferred to a 10 ml ampoule with 5 ml of 6 N HCl. Hydrolysis and amino acid analyses were performed as previously discussed.

RESULTS AND DISCUSSION

Immobilization of Trypsin

Immobilized trypsin on glass, via the azide of the 11-aminoundecanoic acid, retained a portion of its original activity for the hydrolysis of p-tosyl L-arginine methyl ester. Because this experiment represented a preliminary evaluation of the principle of immobilization, no quantitative measurements were made. The column prepared with this form of immobilized trypsin bound soybean trypsin inhibitor (SBTI) as well as ovalbumin and casein when these proteins were percolated separately through the affinity column. Chromatograms are recorded in Figure 3. Nonspecific retention of proteins by the affinity column was a-tributed to ionic forces which exist between amino groups of proteins and the dissociated silanol groups on the glass surface (Messing, 1976).

Trypsin immobilized on agarose beads - Sepharose 4B - retained a large portion of its original activity and exerted specific binding of SBTI as shown by the data presented in Figure 4. Commercial preparations of SBTI are contaminated with other minor inhibitors present in soybean. The presence of small amounts of other proteins than the major inhibitor was verified by SDS-gel electrophoresis. Two

peaks observed during elution of bound SBTI from the affinity column may be attributed to the presence of these proteins with different degrees of association with trypsin.

The amount of trypsin bound to agarose was measured by the difference between the initial amount added and that recovered in the washings. The esterase activity of the immobilized trypsin was measured from the data obtained with a pH-stat using p-tosyl L-arginine methyl ester as substrate. Data relating to these assays are shown in Tables I, II and Figure 5.

Agarose beads both before and after activation and coupling exhibit very little nonspecific adsorption of proteins provided the ionic strength of the buffer is 0.05 M or greater (Cuatrecasas and Anfinsen, 1971). This property, among other considerations (Srere and Uyeda, 1976), made agarose the supporting material of choice for the purpose of this study.

Regained enzymatic activity was calculated by dividing the activity of the immobilized species by that of native trypsin. A value of 19.5% retention was obtained. Activity measurements performed four months later revealed no significant loss of activity (i.e. 19.1%). The preparative scale affinity column made with 100 ml of agarose retained 17.4% of initial activity.

Protein Extraction

The extraction of proteins from dry navy bean with 1 M NaCl pH 7.0 was 45.6% efficient based on the crude protein content of the beans as estimated by Kjeldahl nitrogen determination (N% x 6.25 = protein %) and the dry powder resulting from the extraction. The water soluble fraction - i.e., albumin - compressed 18% and the salt-soluble fraction - i.e., globulins - 82%, respectively, of the extractable proteins (on the Table III). Osborne (1894) reported that a globulin fraction accounted for about 85% of the bean seed protein (Phaseolus vulgaris) - Ishino and Ortega (1975) found that globulins represent 85% of black bean protein.

Heat treated beans yielded only water-soluble proteins - albumins - by extraction with NaCl solution. The globulins which precipitated during dialysis of a NaCl extract of raw beans against water, were absent in the extract of cooked beans, after extensive dialysis. The freeze-dried, water-soluble, extract was low in protein, i.e. 31.5%. Extraction efficiency lowered from 45.6% for raw beans to 6.5% for heat treated beans. Heat causes drastic modifications in the structure of proteins, leading to physical and chemical changes (Joly, 1965). The severe reduction in proteins extractable by NaCl may be attributed to changes in protein solubility.

Separation of Inhibitor

Figure 6 shows the result obtained when the albumin fraction was percolated through the preparative affinity column. The peak eluted with 0.2 M KCl at pH 2.0 represents the inhibitor. This fraction, after dialysis and freeze-drying, contained 59.4% protein and comprised 2.7% of the albumin fraction. The content of inhibitor in the albumin fraction was calculated by the relation between the weight of the freeze-dried inhibitor to the weight of the albumin fraction on protein basis, applied on the column.

Inhibition Measurements

The rate of hydrolysis of casein by trypsin does not follow zero order kinetics under the conditions defined by Kunitz (1949). This aberration has been attributed to limited substrate concentration (Bundy and Mehl, 1958). The modification proposed by Kakade *et al*. (1969) employs 2% casein solution as substrate, instead of 1% in the Kunitz method, and the mathematical transformation of absorbance reading (A) of TCA soluble products to $A^{3/2}$ as proposed by Miller and Johnson (1951). This modified procedure gives a linear relationship between $A^{3/2}$ and enzyme concentration over a large range. The activities of trypsin and α-chymotrypsin in the presence of inhibitors and protein fractions of navy bean, shown in Figure 7 and Appendix Tables I, II, III, were calculated using this transformation.

Inhibition Measurement with SBTI

The amount of inhibitor necessary for complete inhibition of 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. An estimation of the inhibitor molecular weight can be obtained from this plot (Green and Work, 1953a). Based on a molecular weight of 23,300 for trypsin, the method yielded a molecular weight of 12,000 for SBTI. Assuming a molecular weight of 21,500 for the SBTI as reported by Wu and Scheraga (1962) it was calculated that the trypsin was 55.5% active. This correction factor was used in calculating the amount of active trypsin in the inhibition assays. Inhibition of trypsin by SBTI is shown in Appendix Table II.

Inhibition Measurement with NBTI

The navy bean trypsin inhibitor (NBTI) strongly decreased the proteolytic activity of trypsin. The action of NBTI, albumin, globulin and heat treated bean protein on hydrolysis of casein by trypsin is shown in Figures 7a, b, c, d, respectively. Extrapolation to zero activity of the enzyme indicated that 5.850 mg of albumins or 0.515 mg of NBTI was necessary to inhibit 1 mg of trypsin, thus resulting in an increase of 11.3-fold in the specific activity of the isolated inhibitor. The golbulins and heat-treated bean protein had little or no effect on the trypsin activity. The fact that the NBTI is an effective

inhibitor of α-chymotrypsin reported by Bowman (1971) was verified. The NBTI strongly inhibited the hydrolysis of casein by α-chymotrypsin (Appendix Table III).

Soybean trypsin inhibitor strongly reduced the activity of proteinases present in baker's yeast - Saccharamyces cerevisae, and NBTI had little or no effect on such proteinases (Dalilottojari, 1978).

That the trypsin inhibitor is concentrated in the aqueous extract of navy bean was shown by Bowman (1944) and was isolated as a proteinase inhibitor (Bowman, 1971). A water-soluble protein fraction had the highest trypsin inhibitor activity of all fractions obtained from navy beans by Kakade and Evans (1965a). Similar results had been reported for other varieties of Phaseolus vulgaris. Mosolov and Fedurkina (1974) separated trypsin inhibitors from aqueous extracts of the Shirokostruchnaya variety. Seidl et al. (1969) found that a specific trypsin inhibitor from black kidney beans remained in aqueous solution while a non-specific proteinase inhibitor precipitated with the globulin fraction.

The absence of anti-tryptic activity in the proteins extract from heat treated beans agrees with the observation made by Bowman (1944) that the trypsin inhibitor from navy bean is heat labile. Also, Kakade and Evans (1965b) reported that a heat treatment for 121^{o}C for 30 min destroyed nearly all the trypsin inhibitory activity of navy beans.

Assuming a trypsin-NBTI complex formed in 1:1 molar ratio, a molecular weight of 11,900 was obtained for the NBTI from the plot of "remaining activity" vs "inhibitor added". The dissociation constant of the trypsin-inhibitor complex computed from the residual trypsin activity at the equivalence point by the method of Green and Work (1953b) was 7.6×10^{-10}M. Values of the same order of magnitude had been reported for effective inhibitors of trypsin. For example, a value of 2×10^{-10}M was obtained for both pancreatic and soybean trypsin inhibitors (Green, 1953); and a value 5×10^{-10}M for an inhibitor isolated from Phaseolus vulgaris seeds (Mosolov and Fedurkina, 1974).

Molecular Weight Determination by SDS-PAG

Figure 8 shows the electropherogram obtained when the navy bean trypsin inhibitor was applied to 10.8% SDS gel and stained with coomassie blue. The molecular weight estimated from the standard curve of log MW vs. mobility (Figure 9) were 16,600 ± 1,200 and 33,800 ± 3,700 for the major and minor bands, respectively.

Although the molecular weights obtained are informative, the results must be analysed carefully. The basic assumption for the accuracy of molecular weight determinations on SDS gels is that the SDS molecule binds to the protein with a constant ratio and that separation is due to size (Fish et al., 1970; Weber and Kuter, 1971; Svasti and Parrijpan, 1977). Abnormal binding of SDS by some proteins

was shown by Kogen et al. (1972). Anomalous binding of SDS, atypical conformation of the protein-SDS complex, or unusual properties of the native protein-SDS complex maintained in a SDS solution can affect the mobility of proteins in SDS gels (Banker and Cofman, 1972). The ideal situation where 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).

In an attempt to use a protein which resembles the NBTI, at least in respect to origin, soybean trypsin inhibitor was included in the set of known molecular weight proteins. The resulted mobility was much higher than expected for its molecular weight. Considering that the major band of NBTI possessed a mobility very close to that of SBTI, a molecular weight of about 21,000 should be obtained instead of the much lower value which was observed. The molecular weight of SBTI calculated from its relative mobility was 17,400. Because of its peculiar behavior, SBTI was not considered in the linear regression used to derive the calibration curve.

Ferguson plots for NBTI, SBTI and four other proteins are shown in Figure 10. Equations derived from this plot are given in Appendix Table IV. The mobilities (Yo) at 0% total gel concentration (%T) for both NBTI and SBTI were very close, 1.20 and 1.21, respectively. All other proteins had considerable systematic variations in Yo; i.e., Yo

increases with increasing molecular weight (Appendix Table IV).

Equality of Yo's and no systematic trend between Yo and molecular weight or between Yo and retardation coefficient (K_r) is a condition for molecular weight estimates from a single value of %T (Frank and Rodbard, 1975). When these conditions are not achieved, estimation of molecular weight by a relationship between Mw and Kr is preferable since it combines information from several gel concentrations (Rodbard, 1976; Ugel et al., 1971; Kawasaki and Ashwell, 1976).

The plot of molecular weight versus retardation coefficient yielded a linear relationship given by the equation $\hat{y} = 608.98\ x + 3886.40$ $R^2 = 0.9882$, where $\hat{y}$ is the estimated molecular weight and x is 1000 K_r. The molecular weight calculated by this equation for SBTI was 19,300, reflecting the abnormal mobility of this protein in all gel concentrations studied. This equation yielded a molecular weight of 17,650 for the NBTI.

Isoelectric Focusing

The major band of the navy bean trypsin inhibitor showed an isoelectric pH at 4.40, whereas the minor band possessed an isoelectric pH of 4.45. The bands were visible only after staining with coomassie blue since no precipitation occurred at isoelectric pH as it was observed for SBTI.

Amino Acids Determination

Table IV shows the amino acid composition of the navy bean trypsin inhibitor. Amino acids which are subject to degradation during hydrolysis were extrapolated to zero time of hydrolysis. Assuming a first order decomposition, the relationship

$$\log A_0 = \left[t_2/(t_2-t_1)\right] \log A_1 - \left[t_1/(t_2-t_1)\right] \log A_2$$

was applied; where A_0, A_1 and A_2 are the concentrations of each amino acid after 0, 24 and 72 hours of hydrolysis, respectively. Values of 72 h hydrolysis only were considered for those amino acids which are more resistant to hydrolysis.

Important aspects of the amino acid composition of the navy bean trypsin inhibitor were the absence of tryptophan, the low amount of methionine and the high content of half-cystine. The minimum molecular weight, calculated on the basis of methionine as the limiting amino acid, was 12,214. The last two columns of Table IV compare the values obtained from this study with those reported by Wagner and Riehm (1967).

Systematic trends in the amino acid composition pointed out by Birk (1968) for other trypsin inhibitors from plant origin were verified with the navy bean trypsin inhibitor: namely, a remarkably high and constant content of proline, acidic and basic amino acids; a high percentage of serine and threonine associated with a high content of half-cystine; low content or the absence of tryptophan and

the small contribution of methionine.

Table I. Amount of Trypsin Bound to Agarose (20 ml)

	Volume (ml)	Dilution	A_{280}^{1cm}	Amount of trypsin* (mg)
filtrate I	200	1:10	0.205	300.0
filtrate II	100	-	0.030	2.2
filtrate III	100	1:4	0.085	25.2
Total				327.4
Trypsin added				500.0
Bound trypsin				172.6

*Calculated from absorbance by a standard curve at 280 nm.

Table II. Activity of Immobilized Trypsin

Vol. of suspension[a]	div/min[b]	Trypsin (μg)	activity[c]
0.05 ml	19	215.7	88.1
0.07	26	302.0	86.1
0.08	31	345.2	89.8
0.10	39	431.5	90.4
Average			88.6
Trypsin			450.0*

a - total volume of 40 ml (original 20 ml of agarose)

b - taken from initial slopes

c - arbitrarily defined as chart div./min./mg protein

* - average of three demoninations

Table III. Protein Content of Albumin and Globulin Fractions Isolated from Navy Beans

Fraction	g/200 g NB[a]	protein (%)[b]	g prot/200 g NB	% ext. prot.
Albumins	5.15	66.2	3.40	18.0
Globulins	19.30	80.2	15.50	82.0

a - navy bean: 10.9% moisture and 23.2% protein - corrected for moisture

b - content of protein (N x 6.25) in the resulting freeze-dried fractions; average of 4 determinations

Table IV. Amino Acid Composition of Navy Bean Trypsin Inhibitor

Amino acid	μmole/mg prot.			Residues	mole %	Ref. value[3]
	24 h	72 h	Ext.[1]			
lysine	0.518	0.493	0.531	6	5.17	5.27
histidine	0.448	0.420	0.463	5	5.04	4.95
arginine	0.330	0.313	0.338	4	3.30	3.50
tryptophan[a]	-	-	0.0	0	0.00	0.00
aspartic acid	1.459	1.419	1.480	16	14.43	14.39
threonine	0.622	0.575	0.647	7	6.32	6.49
serine	1.492	1.232	1.643	18	16.02	16.64
glutamic acid	0.930	0.886	0.953	10	9.29	8.30
proline	0.723	0.715	0.728	8	7.10	7.79
glycine	0.247	0.243	0.250	3	2.44	2.17
alanine	0.388	0.387	0.388	4	3.77	3.72
½ cystine[b]	-	-	1.267	14	12.36	14.38
valine*	0.193	0.231	0.231	3	2.26	1.04
methionine[b]			0.092	1	0.90	0.62
isoleucine*	0.453	0.495	0.495	5	4.83	4.33
leucine*	0.362	0.374	0.374	4	3.65	2.92
tyronine	0.161	0.131	0.179	2	1.75	1.70
phenylalamine	0.193	0.191	0.194	2	1.90	1.79

[1]Values extrapolated to zero time of hydrolysis or the 72 h results* considered only.

[2]Based on methionine as limiting amino acid.

[3]Wagner and Riehm (1967).

[a]Determined by the procedure of Spies (1967).

[b]Determined as cysteic acid and methionine sulfone, respectively.

Figure 3. Chromatogram showing non-specific binding of glass-trypsin column (3.5x1.25 cm); soybean trypsin inhibitor (SBTI) and ovalbumin-5 $mg \cdot ml^{-1}$; casein-4 $mg \cdot ml^{-1}$. Arrows indicate change in solvent from tris buffer, pH=8.0, to 1 M acetic acid.

Figure 4. Chromatogram showing specific binding of agarose-trypsin column (4.5x1.25 cm); SBTI and ovalbumin-1 $mg \cdot ml^{-1}$. Arrows indicate the same change in solvent as indicated in Figure 3.

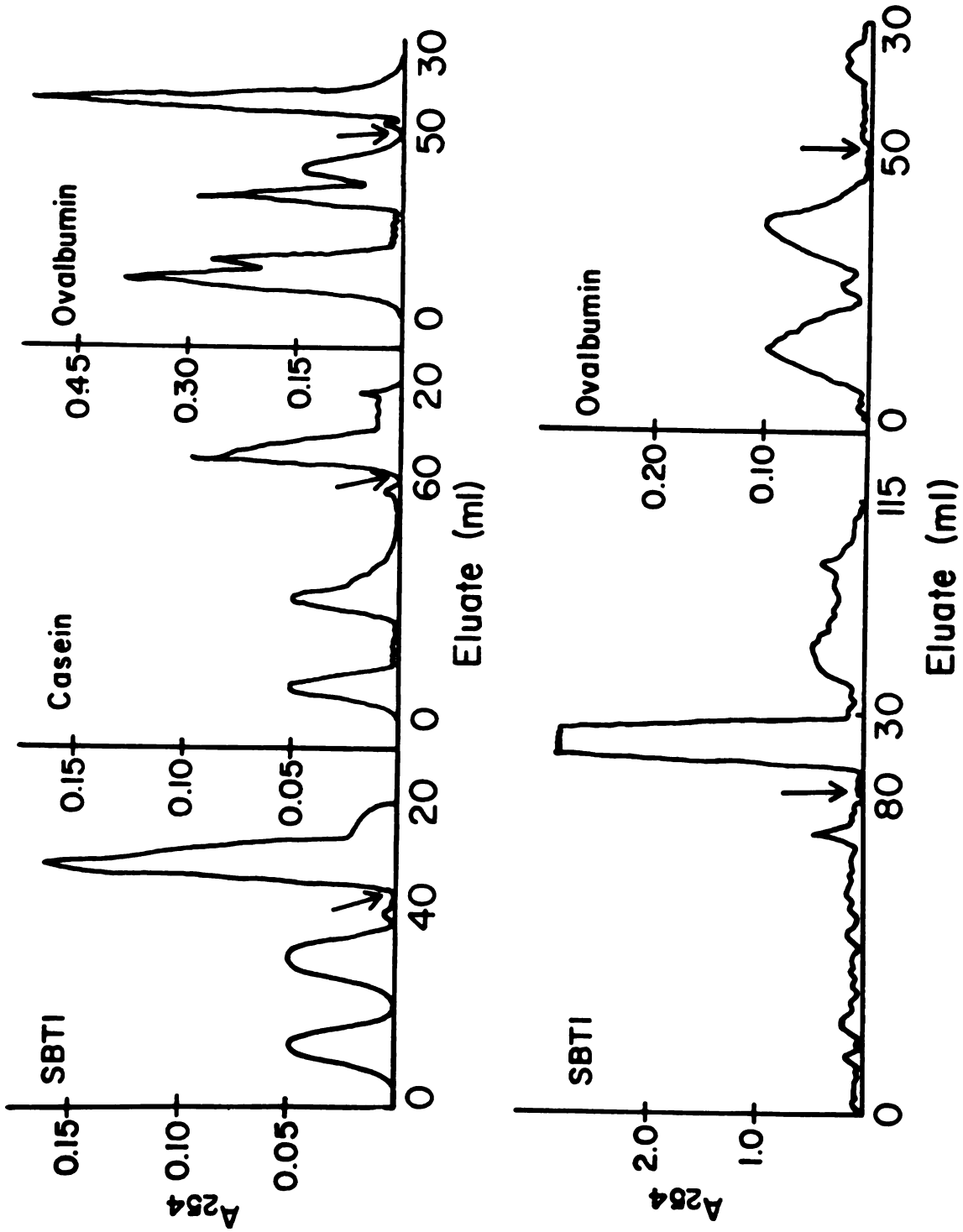

SBTI
Casein
Ovalbumin
A_{254}
0.15
0.10
0.05
0.15
0.10
0.05
0.45
0.30
0.15
0
40
20
0
60
20
0
50
30
Eluate (ml)
SBTI
Ovalbumin
A_{254}
2.0
1.0
0.20
0.10
0
80
30
115
0
50
30
Eluate (ml)

Figure 5. pH stat plot showing the esterase activity of agarose-trypsin (0.05 ml suspension) on p-tosyl b-arginine methyl ester as substrate (pH=8.0; 37°C).

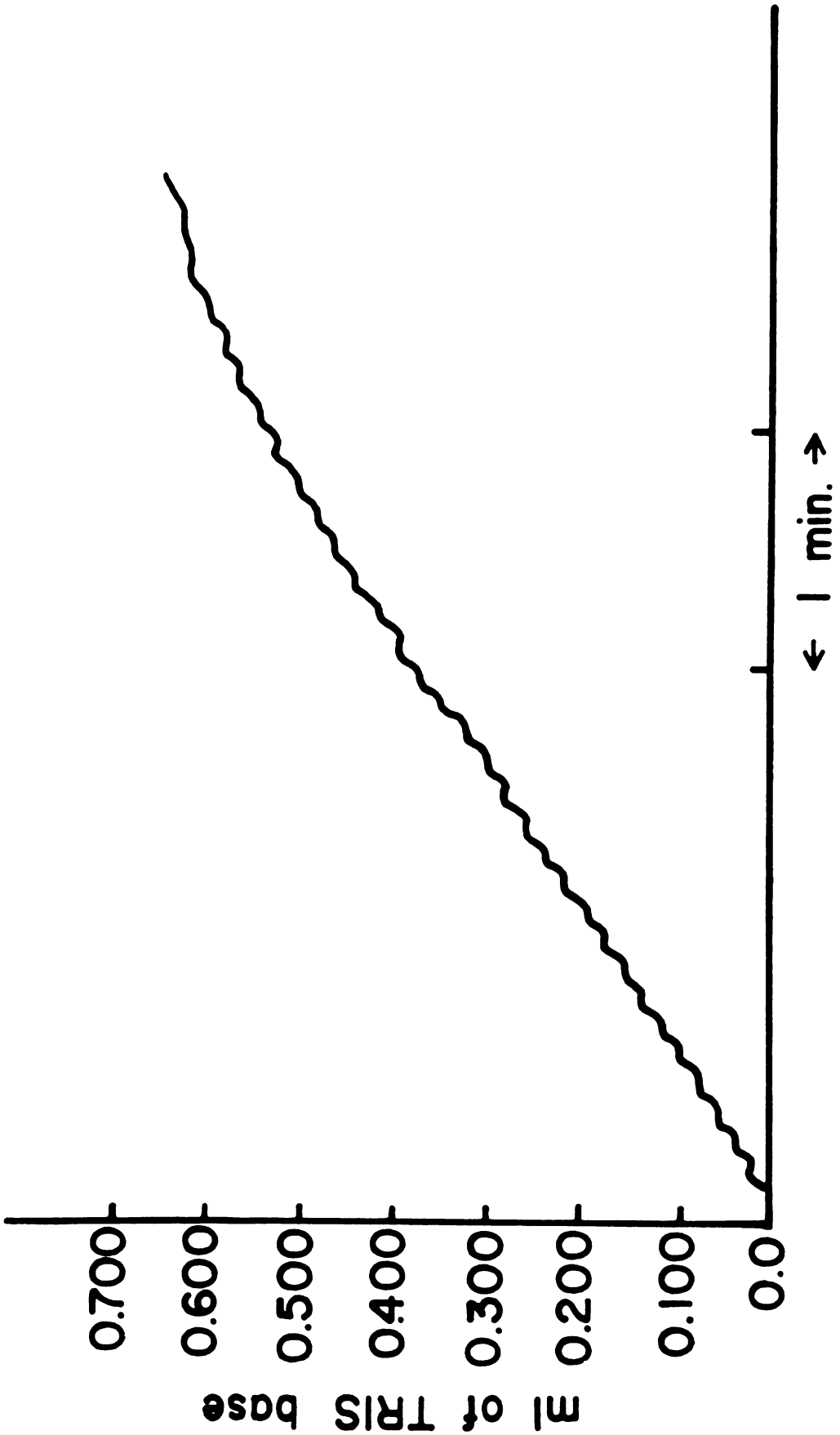

0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.0
ml of TRIS base
↓ 1 min. ↑

Figure 6. Chromatographic separation of navy bean trypsin inhibitor from the albumin fraction column: 18.5x 2.5 cm-aragose-trypsin. Flow rate: 2 ml·min^{-1}, Sample: 50 ml 1% albumin solution in tris buffer pH 8.0. Fractions: 5 ml each. Arrow indicates change in solvent from tris buffer pH 8.0 to 0.2 M KCl pH 2.0.

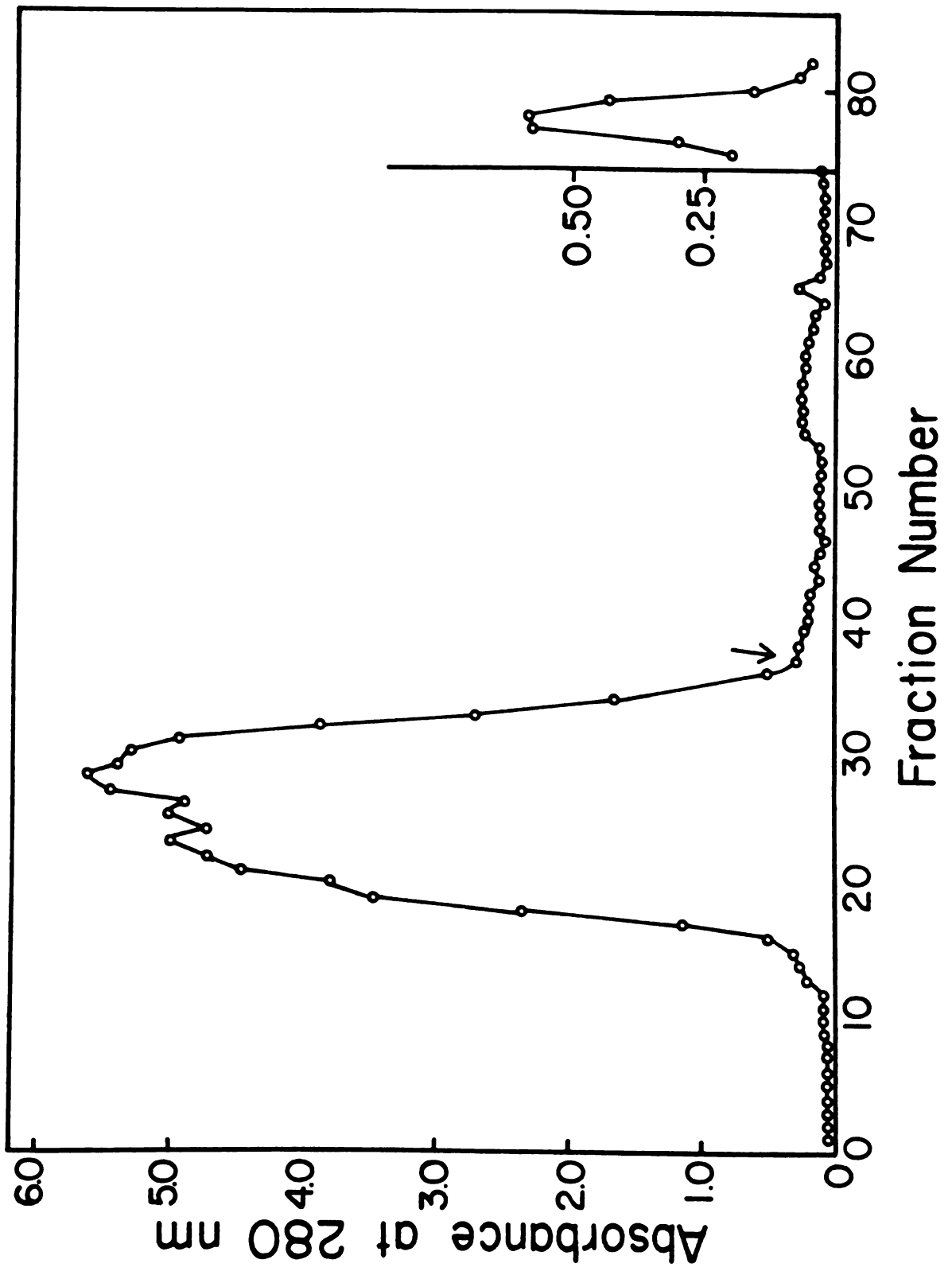
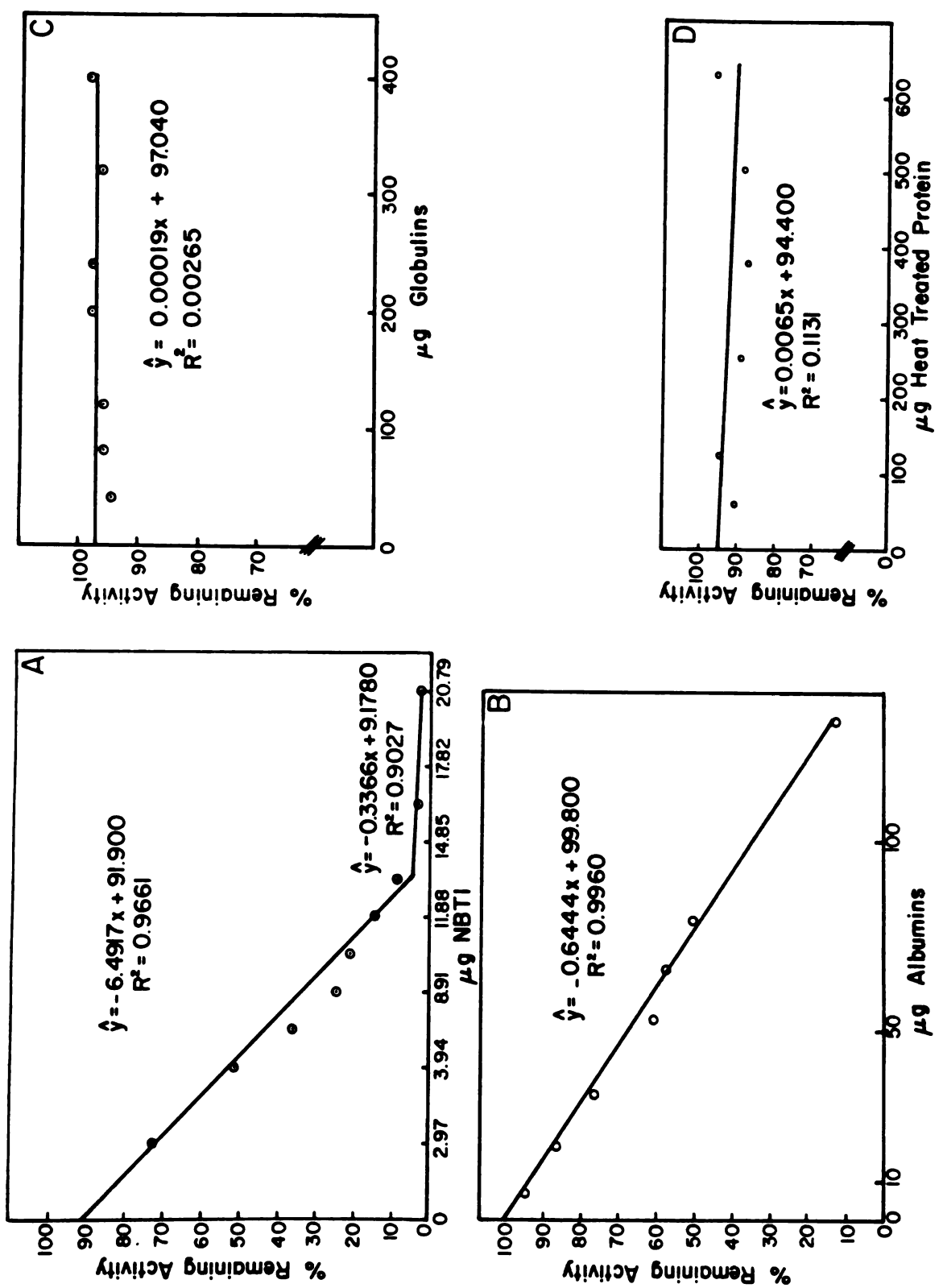

Absorbance at 280 nm
0
1.0
2.0
3.0
4.0
5.0
6.0
0.25
0.50
0
10
20
30
40
50
60
70
80
Fraction Number

Figure 7. Trypsin activity on hydrolysis of casein in relation to the level of: A-navy bean trypsin inhibitor; B-albumin; C-globulins; D-proteins from heat-treated seeds.


A
ŷ= -6.4917x + 91.900
R² = 0.9661
ŷ= -0.3366x + 9.1780
R² = 0.9027
% Remaining Activity
μg NBTI
0
2.97
3.94
8.91
11.88
14.85
17.82
20.79
B
ŷ= -0.6444x + 99.800
R² = 0.9960
% Remaining Activity
μg Albumins
C
ŷ= 0.00019x + 97.040
R² = 0.00265
% Remaining Activity
μg Globulins
D
ŷ= 0.0065x + 94.400
R² = 0.1131
% Remaining Activity
μg Heat Treated Protein

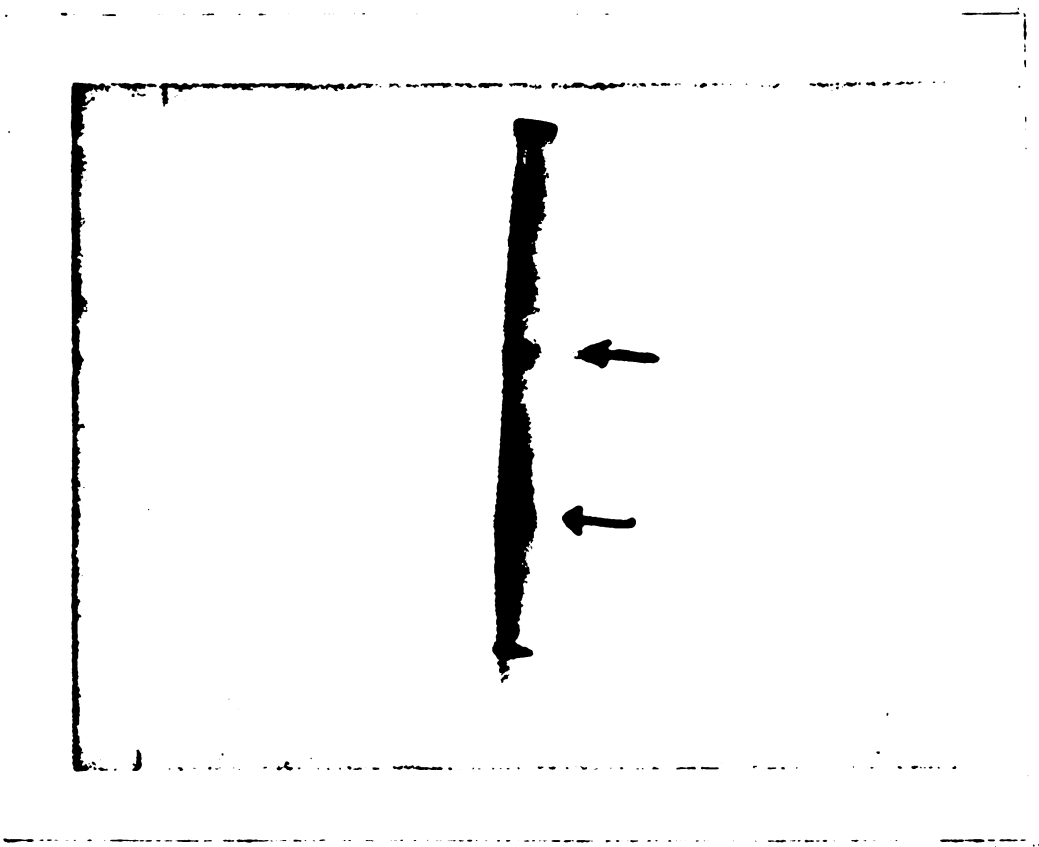
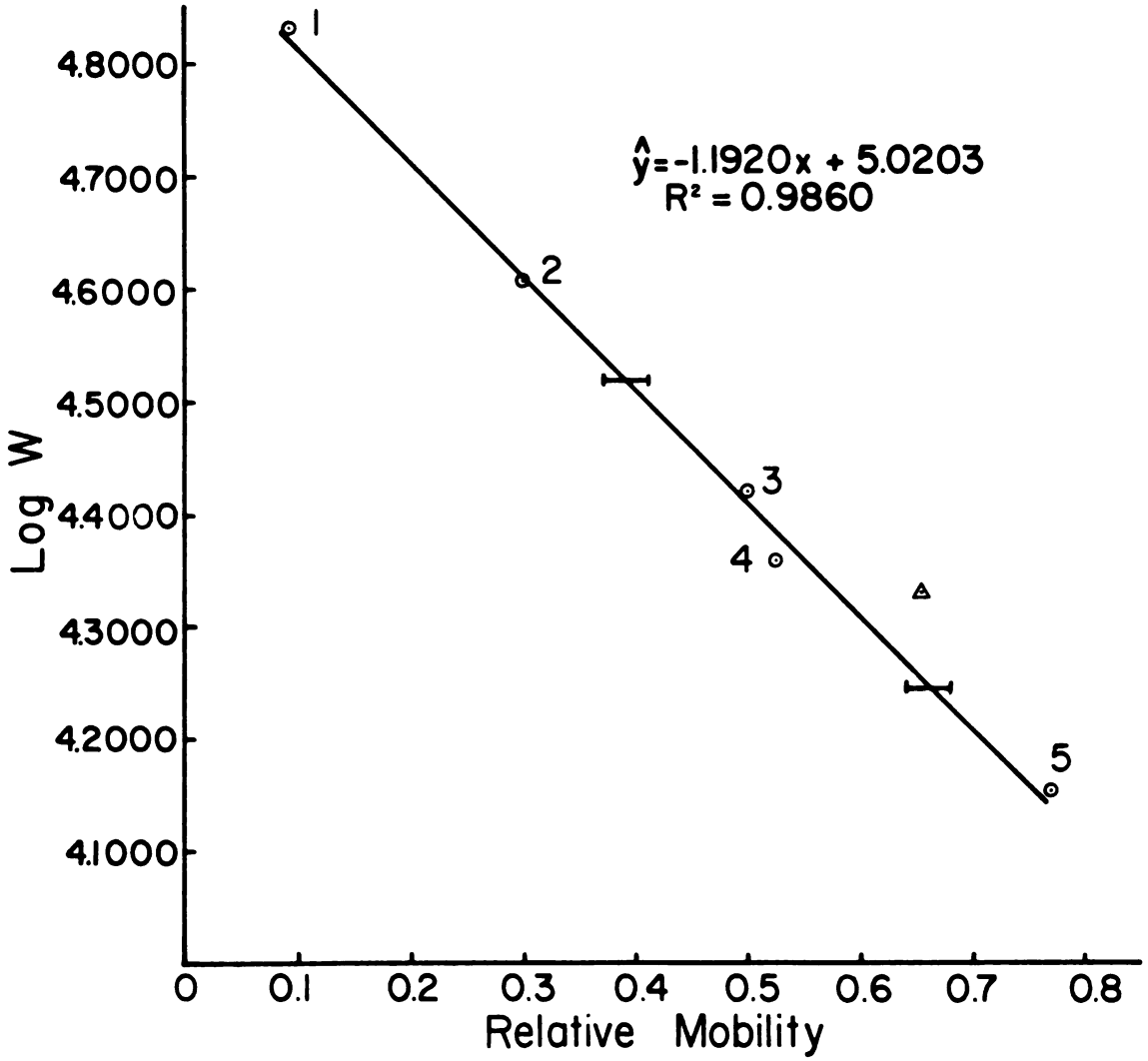
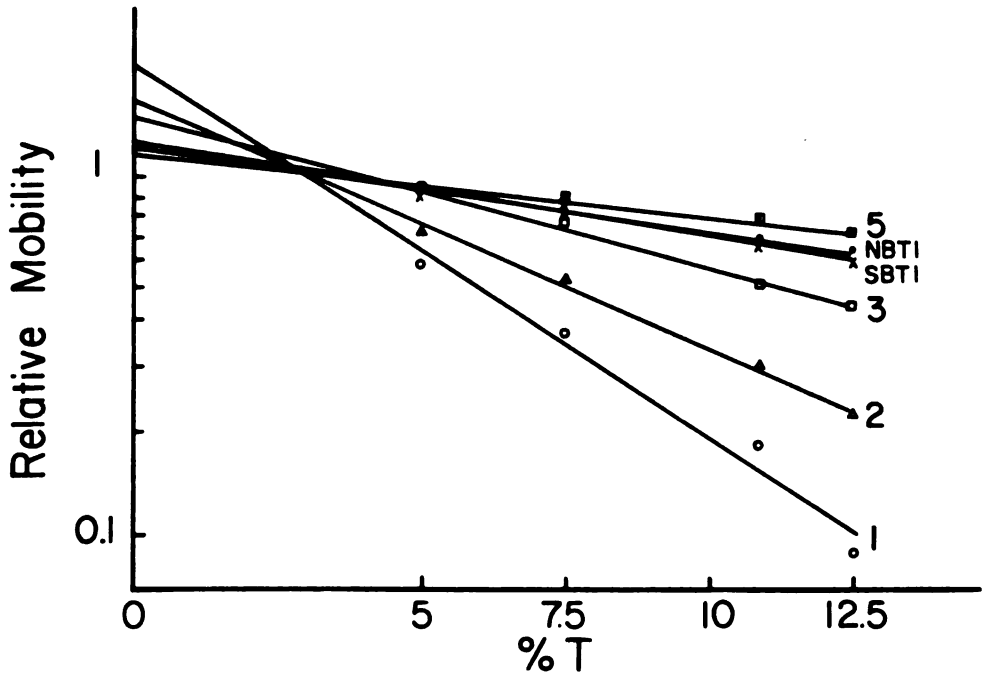


Figure 8. Polyacrylamide-SDS gel (10.8% total concentration) electropherogram of the navy bean trypsin inhibitor.

Figure 9. Standard curve for determination of molecular weight by SDS-PAGE (10.8%). 1 - bovine serum albumin; 2 - ovalbumin; 3 - chymotrypsinogen; 4 - trypsin; 5 - lysozyme. SBTI not included in the linear regression. mobilities for the navy bean trypsin inhibitor. Points are the average of five duplicate gels.

Figure 10. Ferguson plot derived from PAG-SDS electropherograms for navy bean trypsin inhibitor (NBTI), soybean trypsin inhibitor (SBTI) and four reference proteins referred to in Figure 7.


4.8000
4.7000
4.6000
4.5000
4.4000
4.3000
4.2000
4.1000
Log W
$\hat{y}$=-1.1920x + 5.0203
R² = 0.9860
1
2
3
4
5
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Relative Mobility


Relative Mobility
1
0.1
5
NBTI
SBTI
3
2
1
0
5
7.5
10
12.5
%T

CONCLUSIONS

Percolation of albumin fraction from navy beans - Sanilac cultivar - through an affinity column prepared with immobilized trypsin on agarose - Sepharose 4B - and elution with acidic solution yielded a protein fraction which strongly inhibited the enzymatic activity of trypsin and α-chymotrypsin. The navy bean trypsin inhibitor showed two bands under isoelectric focusing. Isoelectric pH was 4.40 for the major band whereas the minor band was at 4.45.

Molecular weights calculated from the relative mobility on SDS-PAG electrophoresis were 16,600 ± 1,200 and 33,800 ± 3,700 for the major and minor bands, respectively. Molecular weight estimated from inhibition measurements with trypsin was 11,900, and the minimum molecular weight, on the basis of methionine as limiting amino acid, was 12,214. The dissociation constant of the trypsin-inhibitor complex computed from the residual trypsin activity at the equivalence point was 7.6×10^{-10} M.

The fact that soybean trypsin inhibitor possessed a relative mobility under SDS-PAG electrophoresis higher than expected for its molecular weight can not be extrapolated for the navy bean trypsin inhibitor. Whether or not the navy bean trypsin inhibitor possess a mobility compatible with its molecular weight could not be established, but the

possibility of a molecular weight of 7,900, as proposed by Whitley and Rowman (1975), can be excluded.

Important aspects of the amino acid composition of the navy bean trypsin inhibitor were the absence of tryptophan, the low amount of methionine and the high content of half-cystine. The amino acid composition is similar to the inhibitor isolated from navy bean seeds by Wagner and Riehm (1967).

The proteins present in the globulin fraction of raw navy beans and the heat-treated bean protein had little or no effect on the hydrolysis of casein by trypsin.

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APPENDIX

Table I. Inhibition of Trypsin Activity by Navy Bean Trypsin Inhibitor

NBTI (μg)	A_{280}*	$A^{3/2}$	% Activity
0	0.552	0.410	100
2.97	0.450	0.302	73.6
4.45	0.405	0.258	62.9
5.94	0.357	0.213	51.9
7.43	0.281	0.149	36.3
8.91	0.220	0.103	25.1
10.40	0.197	0.087	21.2
11.88	0.152	0.059	14.4
13.36	0.112	0.037	9.0
14.85	0.067	0.017	4.1
16.33	0.060	0.015	3.6
17.82	0.060	0.015	3.6
19.30	0.047	0.010	2.4
20.79	0.045	0.009	2.2

*Average of two duplicate determinations.

Table II. Inhibition of Trypsin Activity by Soybean Trypsin Inhibitor

SBTI (μg)	A_{280}*	$A^{3/2}$	% Activity
0	0.552	0.410	100
3.97	0.492	0.345	84.1
7.94	0.430	0.282	68.8
11.94	0.325	0.185	45.1
15.88	0.260	0.133	32.4
23.82	0.040	0.008	1.9
27.79	0.017	0.002	0.5

*Average of two duplicate determinations

Table III. Inhibition of αChymotrypsin Activity by Navy Bean Trypsin Inhibitor

NBTI	A_{280}*	$A^{3/2}$	% Activity
0	0.912	0.871	100
2.97	0.805	0.722	82.9
5.94	0.735	0.630	72.3
8.91	0.620	0.488	56.0
11.88	0.530	0.386	44.3
14.85	0.487	0.340	39.0
17.82	0.422	0.274	31.5
20.79	0.350	0.207	23.8
23.76	0.280	0.148	17.0

*Average of two duplicate determinations

Table IV. Equations Derived from Ferguson Plots[a]

Protein	Mw	Equations Ferguson Plot[b]	R^2	K_r	Y_o
Bovine serum albumin	68000	$\hat{y}$=-0.104x+0.312	0.9724	0.104	2.05
Ovalbumin	45000	$\hat{y}$=-0.067x+0.200	0.9934	0.067	1.57
Chymotrypsinogen	25800	$\hat{y}$=-0.043x+0.171	0.9910	0.043	1.48
Soybean trypsin inhibitor	21500	$\hat{y}$=-0.025x+0.085	0.9868	0.025	1.21
Lysozyme	14300	$\hat{y}$=-0.016x+0.051	0.9422	0.016	1.12
Navy bean trypsin inhibitor	-	$\hat{y}$=-0.023x+0.078	0.9573	0.023	1.20

[a]Validation of Ferguson plot for SDS-PAGE is referred by Rodbard (1976).

[b]Derived from four gel concentrations (5, 7.5, 10.8 and 12.5%). The plot is shown in Figure 10. Points are average of two duplicate experiments except 10.8% gel concentrations which is from five.