Sainfoin trypsin inhibitor : preparation and characterization of the low molecular weight protein
by Walter Frank Baginsky

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
Montana State University

Abstract:
The trypsin inhibitor present in the seeds of the leguminous plant sainfoin (Onobrychis viciifolia, Scop.), variety Eski, was isolated and biochemically characterized. The inhibitor was isolated by affinity chromatography on trypsin-Sepharose 4B and the major isoform of the protein was purified to homogeneity by ion-exchange chromatography on SP-Sephadex C-25. Gel filtration and SDS electrophoresis showed the inhibitor to be a low molecular weight (6400 daltons) protein and to consist of a single polypeptide chain of 57 amino acid residues. Amino acid analysis revealed relatively large amounts of half-cystine (25%), aspartic acid (11%), threonine (11%) and serine (7%) residues. No sulfhydryl, tryptophanyl, methionyl or carbohydrate components were detected. The amino-terminal residue of the inhibitor was determined to be half-cystine. Isoelectric focusing showed an isoelectric point near pH 6.8. The protein was stable to heat, and proteolysis. The inhibitor stoichio-metrically inhibited bovine trypsin in the molar ratio of 1:1 whereas the inhibition of bovine alpha-chymotrypsin was weak and non-stoichiometric. Pancreatic elastase and kallikrein were not inhibited by sainfoin trypsin inhibitor. The purified inhibitor appeared to be an atypical member of the "Bowman-Birk" class of leguminous protein trypsin inhibitors.

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APPROVAL

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Walter Frank Baginsky

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date Feb 27, 1985

To my Dad
Touchdown
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ABSTRACT

The trypsin inhibitor present in the seeds of the leguminous plant sainfoin (Onobrychis viciifolia, Scop.), variety Eski, was isolated and biochemically characterized. The inhibitor was isolated by affinity chromatography on trypsin-Sepharose 4B and the major isoform of the protein was purified to homogeneity by ion-exchange chromatography on SP-Sephadex C-25. Gel filtration and SDS electrophoresis showed the inhibitor to be a low molecular weight (6400 daltons) protein and to consist of a single polypeptide chain of 57 amino acid residues. Amino acid analysis revealed relatively large amounts of half-cystine (25%), aspartic acid (11%), threonine (11%) and serine (7%) residues. No sulfhydryl, tryptophanyl, methionyl or carbohydrate components were detected. The amino-terminal residue of the inhibitor was determined to be half-cystine. Isoelectric focusing showed an isoelectric point near pH 6.8. The protein was stable to heat, and proteolysis. The inhibitor stoichiometrically inhibited bovine trypsin in the molar ratio of 1:1 whereas the inhibition of bovine alpha-chymotrypsin was weak and non-stoichiometric. Pancreatic elastase and kallikrein were not inhibited by sainfoin trypsin inhibitor. The purified inhibitor appeared to be an atypical member of the "Bowman-Birk" class of leguminous protein trypsin inhibitors.

INTRODUCTION

Protein proteinase inhibitors are a diverse group of proteins found throughout the plant and animal kingdoms. They possess the ability to associate reversibly with one or more proteinases to form discrete stoichiometric protein-protein complexes, in which all catalytic functions of the proteinases are competitively inhibited [1]. Not only are protein proteinase inhibitors diverse in number, but they are also different in specificity toward various proteolytic enzymes; that is, some inhibit only one enzyme, whereas others are polyvalent and can inhibit several at the same time [2].

For many years proteinase inhibitors have stimulated the interest of scientists in various disciplines, for a wide variety of different reasons. For example, the majority of initial work pertaining to proteinase inhibitors came from those involved with animal nutrition, who were concerned about the potentially unfavorable dietary effects presented by the proteinase inhibitors found in important food plants and their products [3]. Later the research emphasis turned toward proteinase inhibitor-proteinase interaction, which

offered procedures to further understand basic protein-protein interactions, including those which underlie the mechanism of proteolytic digestion and antigen-antibody complexation [4]. Members of the medical and pharmacological professions felt that the inhibitors had possible potential as therapeutic agents, thus having considerable promise for clinical applications in the field of medicine [5]. In addition, extensive studies have been performed to deduce what physiological functions these inhibitors might have within the living tissues of plants and animals. Research in this latter area has been greatly enhanced by some intriguing but speculative suggestions [6,7,2,8].

The occurrence of proteinase inhibitors in plants has been known since 1938, when Read and Haas reported that an aqueous extract of soybean flour inhibited the ability of trypsin to liquefy gelatins [9]. The Leguminosae, due to its large number of species and their nutritional significance, is one of the most extensively studied plant families regarding proteinase inhibitors. Webster defines a legume as a plant characterized by a "two-valved seed vessel having a row of seeds attached along the seam where the parts join, as in a pod of peas". These nitrogen fixing plants are considered to be protein-rich crops, and offer considerable promise in

supplementing the protein demands of susceptible groups. They have been found to serve a variety of areas which include: human foods, industrial applications, and animal feeds. In the developing countries, there is a lack of animal proteins, or to be more precise, of well-balanced proteins especially those that contribute essential amino acids in the diet [10]. In these developing countries, leguminous seeds are designated as "the meat for poor people" [10]. The developed countries have brought modern technology to bear on the isolation and processing of seed proteins into new foods [11].

Leguminous plant seeds generally account for 20 to 40% of the total protein content of the plant. Furthermore, they have two to three times as much protein as cereal grains. The quantity of lysine was found to be two to three times higher in legume seeds as opposed to cereal grain seeds. This is especially significant since lysine is the essential amino acid that is most lacking in diets comprised largely of cereal grains.

Recently, the National Academy of Sciences, Washington D.C., published a book on tropical legumes that emphasized the importance of legumes as protein resources for the future. The book focused mainly on the largely unstudied, potentially useful legumes of the tropics. The United Nations was also cited as playing a

part through a "Protein Advisory Group," which organizes and distributes scientific literature in this area of concern [12].

As the shortage of protein becomes more severe, it seems feasible that in the future, the supply of protein foods for much of the world's population will be increasingly limited. The majority of available protein is expected to be of plant origin [13]. With this in mind, protein research involving leguminous plants, particularly the seeds, appears to be essential.

During the period 1930-1940, Moses Kunitz accomplished the isolation of the naturally occurring tissue trypsin inhibitor from bovine pancreas, and the trypsin inhibitor from soybean seeds [14]. He established their protein nature, succeeded in crystallizing the inhibitors as well as the trypsin-inhibitor complexes, and pioneered investigations concerning the stoichiometry of the enzyme-inhibitor association. This work represented the first account of productive chemical study on proteinase inhibitors [15]. Remarkably many of the basic concepts set forth by Kunitz are still retained in modern theories on proteinase-proteinase inhibitor interactions. Examples of these concepts include 1:1 (molar) crystallizable complexes of enzyme and inhibitor; a quantitative assay method for

inhibitors; reversible dissociation of enzyme-inhibitor complexes at low pH and the appreciation that the native conformation of the inhibitor is essential in order for proteinase-inhibitor complexes to form [1].

Research on proteinase inhibitors since the Kunitz period has followed a three step attack. Step 1 consists of isolation and purification, step 2 deals with research involving the possible physiological, pharmacological and nutritional significance of the inhibitors, and step 3, the most recent work, involves the understanding of enzyme-inhibitor interaction and specificity at the molecular level [1].

As mentioned above, protein proteinase inhibitors are widely distributed in plants. The majority of these inhibitors are found in the seeds, however they are not necessarily restricted to any one specific part of the plant. For example, in the mung bean the leaves and cotyledons were found to be high in trypsin inhibitor activity, whereas low activity was observed in the stems and roots [16]. The seeds of leguminous plants have long been perceived as excellent sources of protein. Thus, the interest in continued research involving soybean inhibitors as well as other leguminous plant proteinase inhibitors is not surprising.

The proteolytic enzymes found in nature are

comprised of four main groups which are characterized by the nature of their active sites and the reaction mechanism involved. These are the serine proteinases, the sulfhydryl proteinases, the metallo proteinases, and the acidic proteinases. The majority of these enzymes have been shown to be inhibited by proteins isolated from the cells of plants and microorganisms. In some instances, the proteinase inhibitors demonstrate a very narrow span of specificity, being able to inhibit one or possibly two closely related proteinases, while others of broad specificity are able to inhibit a much wider range of diverse enzymes [17]. Studies involving enzymes complexed by the proteinase inhibitors from plants also include digestive enzymes from insects and their larvae that often feed on plants and plant products [18]. These observations proved to be very important in studying the possible physiological role of plant proteinase inhibitors as a defense mechanism against insects.

One of the major ambiguities encountered in determining the specificity of a proteinase inhibitor arises from the fact that some of the early results were based on heterogeneous preparations containing numerous inhibitory proteins [17]. A large amount of the early investigations on the proteinase inhibitors of plant origin concentrated almost exclusively on the inhibition

of trypsin [3]. Subsequent studies have shown that many of the "trypsin inhibitors" also inhibited the enzyme chymotrypsin [19]. Some inhibitors of this type were shown to contain the same reactive site for both enzymes, whereas others showed "double-headedness", the phenomenon that introduced the concept of two independent inhibitory sites per molecule of inhibitor.

There are only a few reports available to indicate that serine proteinase inhibitors also inhibit enzymes of the other three groups. One example is the broad bean inhibitor that inhibits trypsin and chymotrypsin and also strongly inhibits the sulfhydryl enzyme papain [20].

One key aspect of inhibitor-enzyme interaction is the tight rigidity of the reaction sites. Upon enzyme-inhibitor interaction, conformational changes in either enzyme or inhibitor are negligible. The actual interaction appears to represent the classical lock and key model [21]. One intriguing aspect of the inhibitors is their ability to retain inhibitory activity upon replacement of the reactive site residue by another residue. In some situations, a certain specific substitution may lead to the conversion of a strong trypsin inhibitor to a strong chymotrypsin inhibitor. In most other proteins active sites are strongly conserved and substitution of the active site residue in the

molecule leads to total loss of activity [7].

The molecular weights of plant proteinase inhibitors generally fall into two classes, one with a comparatively low molecular weight (8,000-10,000) and the other of higher molecular weight (>10,000). One of the smallest isolated plant inhibitors is the carboxypeptidase inhibitor from potatoes which has a molecular weight of 4,300 [22,23]. In contrast, the largest plant inhibitor known is the papain inhibitor from potato tubers. This inhibitor is a glycoprotein with an estimated molecular weight of 80,000 [24].

Normally, the leguminous plant proteinase inhibitors have a molecular weight of approximately 8,000 with high contents of half-cystine, aspartic acid and serine residues. In addition, inhibitors of this class contain no tryptophan, free sulfhydryl, or carbohydrate moieties. Methonine is generally rare. Larger leguminous proteinase inhibitors characteristically have molecular weight of about 20,000 and low cystine content. These proteins are possibly more rare, as only the trypsin inhibitors from soybean [25] and winged bean [26] have been described.

One of the most outstanding features of the low molecular weight inhibitors is their stability to acids and heat. In addition, many have been shown to be stable

to denaturation by 8 M urea solution [2]. The large percentage of half-cystine residues in these inhibitors confirm extensive cross-linking, an important contributor to stability of proteins [27]. The high molecular weight leguminous inhibitors, such as the Kunitz soybean inhibitor, are low in half-cystine residues and are less stable as compared to the "cystine rich" low molecular weight leguminous inhibitors.

The first plant inhibitor to be characterized fully was soybean trypsin inhibitor (Kunitz). Soybean inhibitor, which has a molecular weight of 22,461, is the most studied of all the leguminous plant proteinase inhibitors and has been used as a model for the studies of other leguminous proteinase inhibitors. The other major inhibitor isolated from soybean is the Bowman-Birk inhibitor that has a molecular weight of 7,975. This low molecular weight inhibitor has also been fully characterized. It shows double-headedness, inhibits one mole each of trypsin and chymotrypsin simultaneously, has seven disulfides, and is resistant to acid, alkali, and heat. The Kunitz inhibitor is single-headed, unstable to acid and heat, and contains only two disulfide bonds [28,29]. In addition to soybean, leguminous plant proteinase inhibitors have been isolated and characterized from lima bean [30], chick pea [31], winged

bean [26], garden beans [32], alfalfa [33], and numerous other leguminous seeds [34-38].

The possible physiological role of proteinase inhibitors is described in reviews by Ryan [39] and more recently by Richardson [17]. In the broad sense, their purpose is obvious- inhibition of proteolytic activity. Nevertheless, specific functions of the inhibitors are still unresolved, particularly so in the case of plant inhibitors.

The general consensus on the physiological function of secretory pancreatic inhibitors, which occur in all mammals, is one of prevention of premature activation of zymogen activity of the digestive enzymes [40]. It has also been found that individuals deficient in alpha-1-proteinase inhibitor develop pulmonary emphysema rapidly [41]. Emphysema is the result of increased turnover of lung connective tissue proteins, primarily elastin. Research has shown that alpha-1-proteinase inhibitor inhibits neutrophil elastase at a rate ten-fold greater than of any other proteinase tested [42]. Additionally, the large quantities of proteinase inhibitor in mammalian blood are believed to moderate the reactions leading to blood clotting [43].

Several possible roles have been suggested in regard to the function of inhibitors of plant origin. Some of

the plant proteinase inhibitors have the capability of inhibiting endogenous proteolytic enzymes of the plant from which they were isolated, thereby controlling protein turnover and metabolism. However, the majority of the plant inhibitors studied apparently do not inhibit their own proteinases [39]. Alternatively, Pusztai has suggested that the proteinase inhibitors may act as sulfur depot proteins since many contain a relatively large number of cystine residues [8]. Other researchers feel that due to the leguminous plants existing in symbiosis with root-associated bacteria, the suggestion of a possible function of the plant inhibitors is to prevent the plant from being engulfed by the symbiotic bacteria. In addition, the inhibitors may in fact protect the plant tissue at the colonization site against the action of bacterial proteinases [2].

Recent developments strongly emphasize the possibility of plant proteinase inhibitors serving as a defense function against insect attack by inhibiting insect proteinases [6,39,44]. A major advance in this field was made in 1972 when Green and Ryan [18] showed that wounding of the leaves of potato or tomato plants by adult Colorado potato beetles or their larvae produced a rapid increase of proteinase inhibitor throughout the plants tissues. It was later shown that the accumulation

of the inhibitor was directly due to the wounding of the leaf, since any type of crushing would cause the same induction. Results of the above study on tomato plants indicated the probability of a chemical signal that initiates the increase in inhibitor concentration within the plant. Later research showed that this chemical signal was a substance produced or released near the wound. The substance in question has been given the name proteinase inhibitor inducing factor (PIIF), and has been partially purified [44,45]. Earlier research proved that the release and transport of PIIF is both light and temperature dependent [46]. These characteristics along with the increased accumulation of proteinase inhibitor in leaves, as the result of insect attack, indicate that the wounding of a single leaf sets off an "immune-like" response in the plant [47]. It is believed that knowledge of this response may play an important role in the design of new approaches to biological pest control.

Insect pest control has become one of the most important and controversial problems facing the agricultural community of the world today. In the United States insects cause estimated annual crop losses of 15% [48]. Chemical control of insect pests is expensive and often environmentally and politically unacceptable [49,50]. Therefore, it appears favorable to better our

understanding of plant proteinase inhibitors in the hope that ongoing research may one day uncover a biological "built-in insecticide" to combat insects.

The use of plant proteinase inhibitors as valuable laboratory tools will continue to expand. Researchers have successfully employed the inhibitors in the area of affinity chromatography by covalently attaching the inhibitor to inert water insoluble polymeric supports, thus purifying specific proteinases [51,52]. A second application of plant inhibitors was shown by separating proteins based on biospecific interaction with a soluble affinity reagent in conjunction with ultrafiltration [53]. These findings provide examples of the usefulness of the plant proteinase inhibitors as isolation and purification tools. Therefore, in order to fully understand and utilize the capability of the proteinase inhibitors of plants, further research is essential.

GOALS AND OBJECTIVES

The principal objective of this research is the biochemical purification and characterization of the major trypsin isoinhibitor from the seeds of the leguminous plant sainfoin (*Onobrychis viciifolia, Scop.*). Results of the work are to be written into manuscript form and published in the scientific literature.

MATERIALS AND METHODS

Materials

Sainfoin seeds (Onobrychis viciifolia, Scop., variety Eski) were generously supplied by Dr. R.L. Ditterline, Montana State University. Alpha-chymotrypsin was obtained from Worthington. Trypsin (DPCC treated, type XI), pepsin, kallikrein, elastase, alpha-N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE), and alpha-N-benzoyl-L-tryrosine ethyl ester (BTEE) were from Sigma. Sepharose 4B, Sephadex G-50, and sulfopropyl Sephadex C-25 were from Pharmacia. Protein markers for molecular weight determination were purchased from Pharmacia and BRL. Enzyme grade urea was purchased from BRL. All other chemicals were pure or reagent grade. Distilled water was used throughout.

Purity Determination of Trypsin and Alpha-Chymotrypsin

Trypsin used in this research was purified by affinity chromatography on soybean trypsin inhibitor (STI) that was covalently attached to Sepharose 4B by the cyanogen bromide coupling method [54]. A 1.5 x 20 cm

column of STI-Sepharose 4B was equilibrated with three bed volumes of .01 M Tris-HCl buffer containing .01 M calcium chloride and .15 M sodium chloride, pH 7.5. Approximately 25 mg of trypsin (initially dissolved in .005 M HCl, .005 M calcium chloride and subsequently brought to pH 7.5 with 1 M Tris-HCl, pH 7.5) was applied to the column at a flow rate of 20 ml/hr at room temperature. The column was then washed with Tris buffer, pH 7.5, until the 280 nm absorbancy of the effluent returned to zero. Adsorbed trypsin was removed from the column by elution with .001 M HCl that contained .01 M calcium chloride and .15 M sodium chloride. The active fraction was collected in one test tube at 0°C. The functional normality of the collected fraction was then determined by active site titration with p-nitrophenyl-p′-guanidinobenzoate [55]. Alpha-chymotrypsin activity was analogously determined by the method of Kezdy and Kaiser [56].

Protein Concentrations

Trypsin and alpha-chymotrypsin concentrations were determined from their absorbance and established extinction coefficients [57].

Purification of Sainfoin Trypsin Isoinhibitors

Extraction. Dehulled and finely ground sainfoin seeds (280 g) were stirred over night at 4°C in one liter of pH 7.0, 0.01 M potassium phosphate buffer containing 0.01 M ascorbic acid, 0.15 M sodium chloride and .001 M sodium azide. The extract was separated from insoluble material by compression through cheesecloth. Trichloroacetic acid was then added to 2.5% (w/v) and the extract was stirred one hour at 22°C. After standing for 20 min the suspension was centrifuged at 10,000 x g for 30 min. The supernatant solution was adjusted to pH 7 with 10 M sodium hydroxide.

Affinity chromatography. Trypsin-Sepharose 4B was prepared using cyanogen bromide-activated Sepharose 4B and bovine trypsin according to the method of March [54]. The affinity matrix (100 ml) was added to the supernatant solution in batchwise fashion, and allowed to stir at room temperature for 2 hours. The adsorbed affinity matrix was collected on a Buchner funnel and packed into a 2.5 X 25.0 cm column. The column was washed with 0.01 M potassium phosphate buffer pH 7.0, containing 0.15 M sodium chloride, until the effluent had 280 nm absorbance of less than 0.02. Adsorbed inhibitor was then eluted from the column with 0.1 M beta-alanine

buffer, pH 2.5, that contained 0.15 M sodium chloride. The trypsin inhibitor fraction was concentrated to about 5 mg/ml by ultrafiltration in an Amicon ultrafiltration cell using a UM2 membrane.

Ion exchange chromatography. The concentrated sample was dialyzed in 0.01 M sodium citrate, pH 4.0 and then applied to a 1.5 x 50.5 cm column of sulfopropyl Sephadex C-25, previously equilibrated with pH 4.70, 0.01 M sodium citrate buffer. Buffer was pumped through the column at a flow rate of 31 ml/hr by means of a peristaltic pump. The column was developed with two column volumes of initial buffer, followed by a linear gradient of 0.03-0.20 M sodium ion produced from 300 ml each of initial buffer and the initial buffer that contained 0.17 M sodium chloride. The column effluent was monitored at 230 nm and 2.5 ml fractions were collected. Peak fractions were individually pooled, desalted by dialysis against water, and concentrated by ultrafiltration over a UM2 membrane.

Characterization of Inhibitor II

Isoelectric focusing. Isoelectric focusing was carried out in 0.5 X 10 cm glass tubes, with a Hoefer DE 102 tube gel unit, according to the method of Wrigley

[58]. Protein samples were focused in 6% total acrylamide in a pH 3-10 gradient. Bands were visualized by soaking the gels in 12% (w/v) trichloroacetic acid.

Amino acid analysis. Amino acid analyses were carried out on a Beckman 120C amino acid analyzer coupled to an Infotronics CRS110A digital integrator according to Spackman et al. [59]. Protein samples were hydrolyzed with constant boiling HCL in evacuated, sealed hydrolysis tubes for 24 hr at 110°C. Hydrolyzates were then dried, dissolved in sodium citrate sample buffer (pH 2.20) and analyzed.

Gel filtration. Molecular-sieve chromatography was performed on a 0.9 x 1000 cm column of Sephadex G-50 following the method of Andrews [60]. The column was equilibrated with .05 M Tris-HCl buffer, pH 7.5, that contained 0.1 M sodium chloride at a flow rate of 20 ml/hr at 22°C. Calibration standards were bacitracin (1,450), bovine pancreatic trypsin inhibitor (6,500), cytochrome C (12,400), myoglobin (17,800) and ovalbumin (70,000).

Urea SDS- polyacrylamide gel electrophoresis. Urea SDS-polyacrylamide gel electrophoresis was carried out by a slight modification of the procedure of Shapiro et al.

[61]. Electrophoresis was performed using a 14 x 16 cm separating gel slab of 15% polyacrylamide containing 0.1 M sodium phosphate pH 7.2, 0.1% SDS and 6 M ultra pure urea. The stacking gel consisted of 3.5% polyacrylamide, and had buffer conditions identical to the separating gel. Tank buffer was 0.1 M sodium phosphate, pH 7.2, that contained 0.1% SDS.

All samples (10 ug) were diluted separately into 40 ul of sample buffer consisting of 0.01 M sodium phosphate pH 7.2, 7 M urea, 1% SDS, 1% 2-mercaptoethanol, and 0.01% bromphenol blue. Prior to application the calibration standards were heated at 95°C for 2 min while the inhibitor sample was incubated at room temperature for 15 min. The gels were run at 5 volts/cm at room temperature until the bromphenol blue tracking dye reached the bottom of the gel (approximately 20-22 hr). After electrophoresis was completed, gels were placed in staining solution containing 0.1% Coomassie Blue G-250, 25% isopropanol, 10% acetic acid and 0.1% cupric acetate for 7 hr. Destaining was performed for 24 hr in methanol/acetic acid/water (5:7:88 by vol). Inhibitor molecular weight was estimated from standard curves derived from the mobility of the reduced calibration standards relative to the log of their molecular weight.

Protein transfer from polyacrylamide gel to nitrocellulose filters. The method of Towbin [62] was used to electroelute proteins from polyacrylamide gel slabs onto nitrocellulose filter paper of 0.2 um porosity (Shleicher & Schuell, Keene NH). The transfer apparatus was a Hoefer TE series transphor unit, and all transfers were performed at 13°C. The gel was transferred in buffer (.025 M Tris, .192 M glycine, 20% methanol v/v, pH 8.3) with the nitrocellulose on the anodic side of the gel for 2 hr at a current of 1.0 amps. Following the transfer, the nitrocellulose paper stained for 5 min. at room temperature with 0.2% Amido-Black in 7% acetic acid and destained in acetic acid/methanol/water (7:5:88 v/v).

Absorption spectrum and extinction coefficient. The ultaviolet absorption spectrum of sainfoin inhibitor II was determined using a Varian 635 dual-beam spectrophotometer equipped with a Varian Techtron recorder.

The concentration of a neutral solution of inhibitor II was determined by quantitative amino acid analysis. The $E^{1\%}280$ was calculated from the concentration of protein in the sample from its the absorbance at 280 nm.

Molecular Stability of Inhibitor II

Resistance toward pepsin. The effect of pepsin on sainfoin inhibitor II was examined. Inhibitor II was mixed with pepsin to give a final concentration of 5.0 mg/ml inhibitor and 0.5 mg/ml pepsin. The mixture was incubated at 37°C. Aliquots containing 10 ul were removed at various time intervals and mixed separately with 3 ml of 0.05 M borate buffer pH 9.1, followed immediately by the addition of 1 ml of a solution containing .02% fluram (w/v) in acetone. Fluorescence was determined with the Varian instrument using exciting light at 400 nm. As a control, bovine serum albumin was treated under identical conditions.

Heat treatment. Heat stability of inhibitor II was determined by the following method. The inhibitor sample was incubated at 95°C for 3 hr. At increasing time intervals, aliquots of the inhibitor solution were withdrawn and assayed for trypsin inhibition. Inhibitor II incubated at 22°C was used as a control.

Specificity of Trypsin Inhibitor II

Trypsin and chymotrypsin esterase activity were measured spectrophotometrically using enzyme rate assays

described by Walsh and Wilcox [57]. All experiments were carried out at room temperature. The substrate concentrations used for the inhibitory assays were 5.25 X 10^{-4} M alpha-N-benzoyl-L-arginine ethyl ester (BAEE) and 2.91 X 10^{-4} M alpha-N-benzoyl-L-tyrosine ethyl ester (BTEE). Elastase and kallikrein activity were determined in a similar fashion utilizing substrates N-benzoyl-L-alanine-methyl ester and BAEE, respectively [63,64].

The inhibitory activity of sainfoin inhibitor II was obtained from the change in slope as recorded spectrophotometrically from a series of reaction mixtures containing enzyme and varied amounts of inhibitor. The reaction mixture involving trypsin inhibition contained an aliquot of inhibitor (0-45 ul containing 0-21 ug protein) diluted to 200 ul with 0.05 M Tris-HCl, 0.01 M calcium chloride, 0.15 M sodium chloride, pH 7.80 and 20 ul (7 ug) trypsin. The chymotrypsin activity assay incorporated an aliquot of inhibitor (0-65 ul containing 0-30 ug protein) diluted to 200 ul with 0.05 M Tris-HCl, 0.01 M calcium chloride, 0.15 M sodium chloride, pH 7.8 and 10 ul (7 ug) alpha-chymotrypsin. Reaction solutions were allowed to incubate 10 min at room temperature. At this time, 100 ul aliquots were withdrawn and assayed using the appropriate substrates.

Determination of Stoichiometry of Inhibition by Inhibitor II

Purified sainfoin trypsin inhibitor II was adjusted to pH 7.5 and repurified on a 0.5 x 3 cm column of trypsin-Sepharose 4B in order to assure 100% active inhibitor for use in the stoichiometry determination. The column was equilibrated at room temperature with two bed volumes of pH 7.5, .01 M Tris-HCl buffer prior to application of inhibitor sample. The column was then washed with 10 volumes of the same buffer followed by release of the active inhibitor upon elution with 1 mM HCl.

Data points obtained from the enzyme rate assay of inhibited enzyme were treated by least squares analysis which resulted in a x-intercept value indicative of the quantity of inhibitor which inhibits 100% of enzyme. The stoichiometry of inhibition is then determined by the molar ratio of inhibitor to enzyme at the point of 100% inhibition.

Chemical Modification of Inhibitor II

Amino-terminal. The amino-terminal residue of sainfoin inhibitor II was deduced by a procedure similar to that of Namen and Hapner [65]. A sample

containing .45 mg of inhibitor II was dialyzed against 0.1 M sodium bicarbonate pH 8.4 for 5 hr. After dialysis a 40-fold molar excess of iodoacetic acid over total amino groups was added. The solution was adjusted to pH 8.4 and incubated at 54°C for 19 hr. Excess reagents were removed by dialyzing against water for 5 hr. The inhibitor sample was then evaporated to dryness, hydrolyzed with 1 ml of 6 N HCl at 110°C for 24 hr and analyzed on the amino acid analyzer. The results were compared to the amino acid analysis of a comparable inhibitor sample not treated with iodoacetic acid. Loss of an amino acid residue was assumed to be due to alkylation of the nitrogen-terminus.

Carboxymethylation. The reduction and carboxymethylation of sainfoin inhibitor II was performed according to the method of Thomas et al. [66]. The lyophilized protein was dissolved in a solution consisting of 6 M guanidinium chloride and 0.1 M Tris, pH 9.5. Reduction was carried out by the addition of a 10-fold molar excess of dithiothreotol. The solution was incubated under nitrogen, in the dark, for 5 hr. The inhibitor was then carboxymethylated with a 3-fold molar excess of recrystallized iodoacetic acid. The reaction was quenched after 5 min by the addition of a 10-fold molar excess of 2-mercaptoethanol. Excess reagents were

removed by dialysis in water at 4°C. After dialysis, the carboxymethylated inhibitor was lyophilized, hydrolyzed, and analyzed for S-carboxymethylcysteine with an amino acid analyzer.

Performic acid oxidation. Sainfoin trypsin inhibitor was oxidized the method of Hirs [67] and subjected to amino acid analysis for determination of cysteic acid.

RESULTS

Purification of Sainfoin Trypsin Isoinhibitors

Fig. 1 illustrates the elution profile obtained by affinity chromatography of the initial crude extract on trypsin-Sepharose 4B. Equilibrating buffer was used to wash nonadsorbed inactive proteins and pigments from the column. Upon the addition of the pH 2.5 buffer, the adsorbed inhibitors dissociated from the trypsin-Sepharose 4B and eluted as a sharp peak of absorbancy. BAEE assay of collected fractions showed no inhibitory activity prior to the addition of pH 2.5 buffer. The peak containing the active protein was pooled and concentrated. The buffer salts were removed by dialysis of the active fraction in 0.01 M sodium citrate, pH 4.0.

The dialyzed sample was subjected to ion-exchange chromatography on sulfopropyl-Sephadex C-25 at pH 4.70. As shown in Fig. 2, four peaks of absorbancy were obtained. Peaks 1 and 2 emerged from the column with initial buffer, while peaks 3 and 4 were eluted with an increasing gradient of sodium chloride. The major isoinhibitor (peak 3) eluted at approximately 0.08 M NaCl and showed affinity for the ion exchange resin

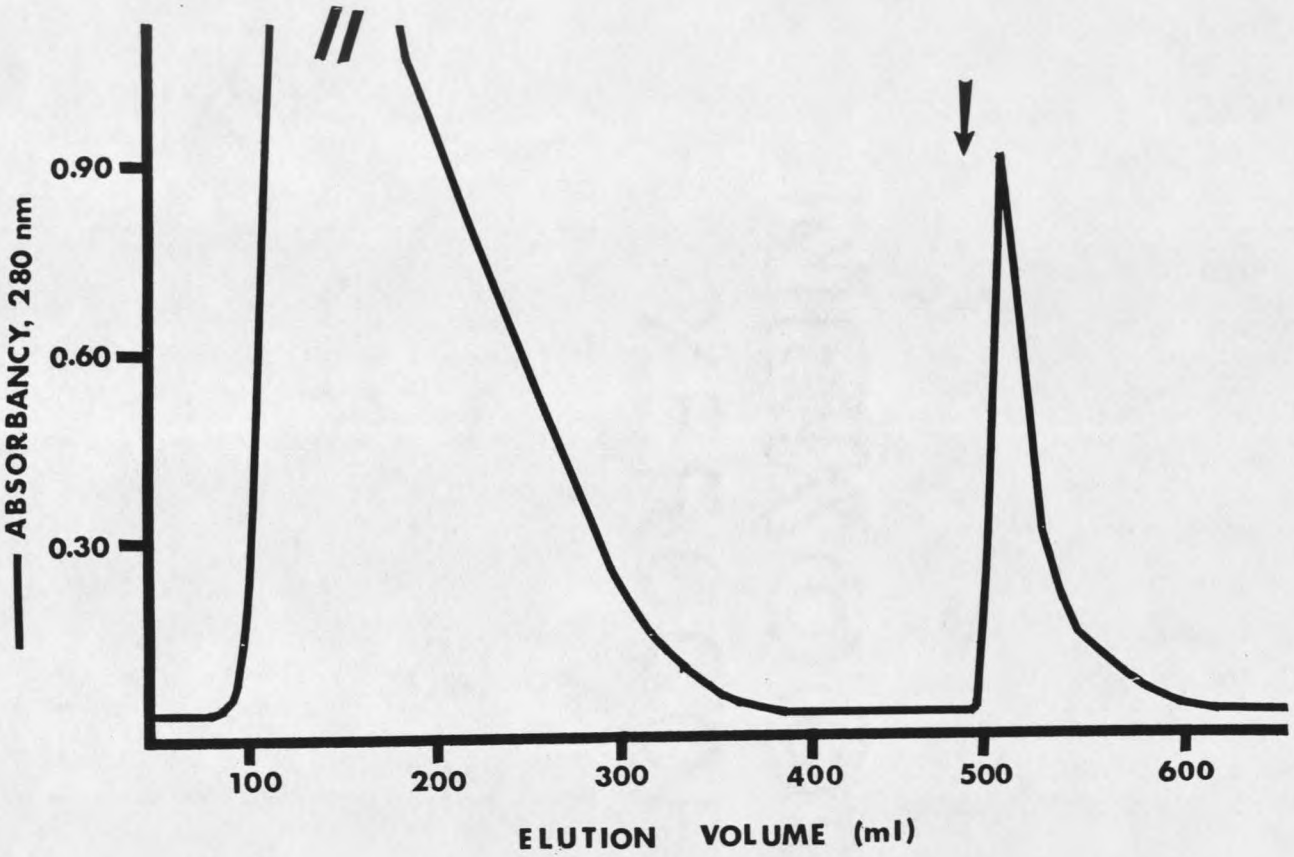

Figure 1. Affinity chromatography of sainfoin trypsin inhibitor. Elution of crude sainfoin trypsin inhibitor is with 0.1 M beta-alanine (arrow).

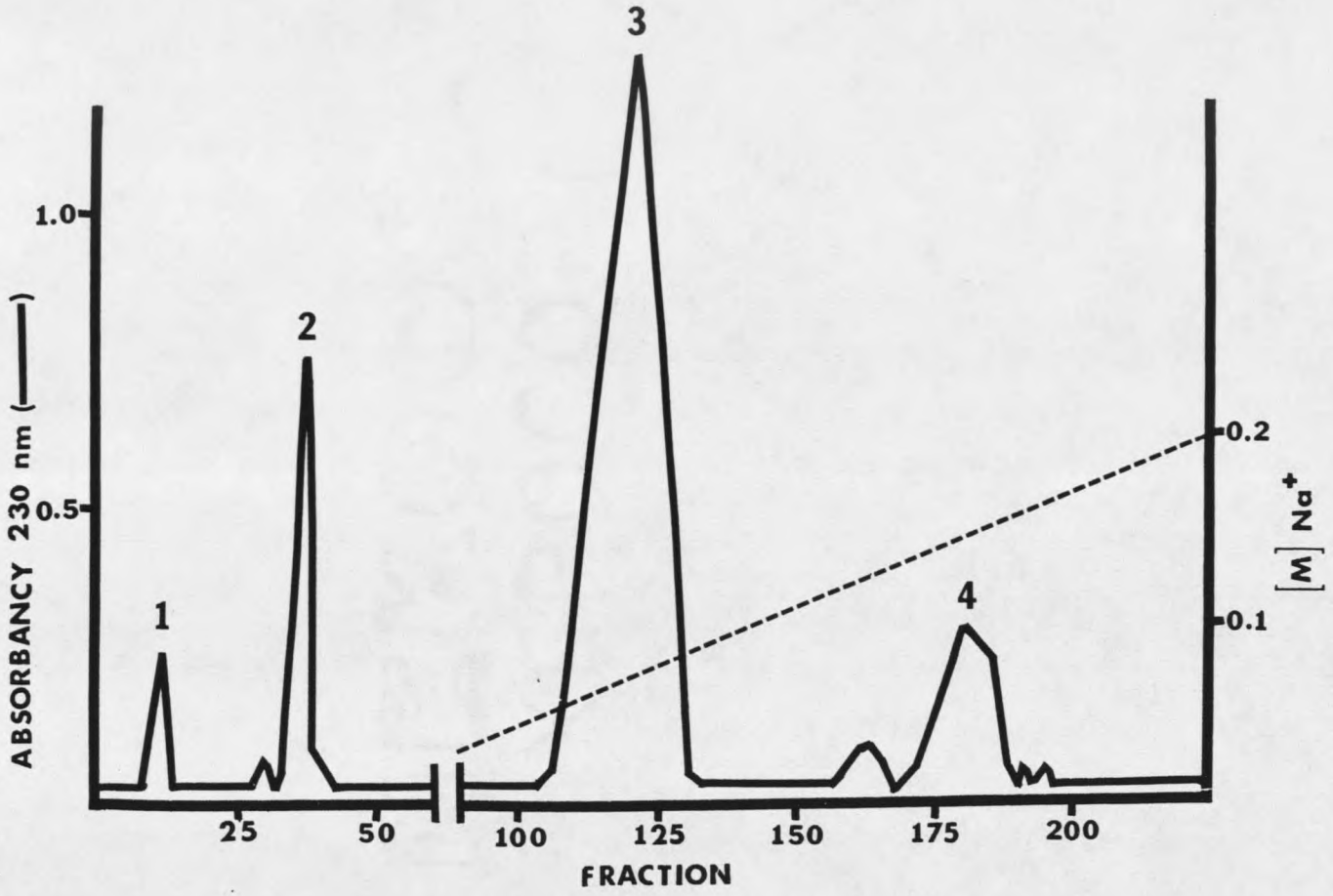

Figure 2. Ion exchange chromatography of sainfoin trypsin inhibitor. Fractions indicated by number were pooled.

intermediate of that of peaks 2 and 4. Peaks 2, 3, and 4 showed strong inhibitory activity against trypsin, whereas fraction 1, not retained by the column, showed only trace amounts of inhibitory activity. Each peak was pooled as indicated and concentrated by ultrafiltration. Peaks 2, 3, and 4 were designated as the trypsin inhibitor fractions I, II and III, respectively. The yields of the individually pooled peaks obtained from the SP-Sephadex C-25 ion exchange column are summarized in Table 1. The major isoinhibitor II was twice as abundant as the other isoforms and represented about 35% of total crude inhibitor.

Table 1. Yields of the Peaks From the SP-Sephadex C-25 Ion Exchange Chromatography.

Peak	Protein (mgs)	Yield[a] (%)
1	2.8	19.4
2	0.8	5.6
3	5.1	35.4
4	2.6	18.1
Total	11.3	78.5

[a]Based on total protein applied to the column.

Biochemical Characterization of Inhibitor II

Homogeneity. Determination of purity was performed by isoelectric focusing and the results are shown in Fig. 3. Inhibitor II (major isoform) produced a single homogeneous band that focused near pH 6.8 (estimated from calibration protein, not shown). In addition to inhibitor II, inhibitors I and III were also homogeneous, and focused near pH 8.1 and pH 4.3, respectively. Further supporting data for the homogeneous state of sainfoin inhibitor II was obtained by urea SDS-polyacrylamide gel electrophoresis. Figure 4 shows the results. Inhibitor II migrated as a single band near the position of bovine pancreatic trypsin inhibitor which has a molecular weight of 6,500. If the inhibitor II was heated prior to application to the gel slab, smeared and diffuse staining occurred indicating molecular degradation.

Molecular weight. The molecular weight of inhibitor II determined by gel filtration on a calibrated column of of Sephadex G-50 was found to be 6,500 as shown in Fig. 5. The inhibitor eluted as a single peak with identical elution time as bovine pancreatic trypsin inhibitor (M.W. 6,500). Amino acid analysis (see later)

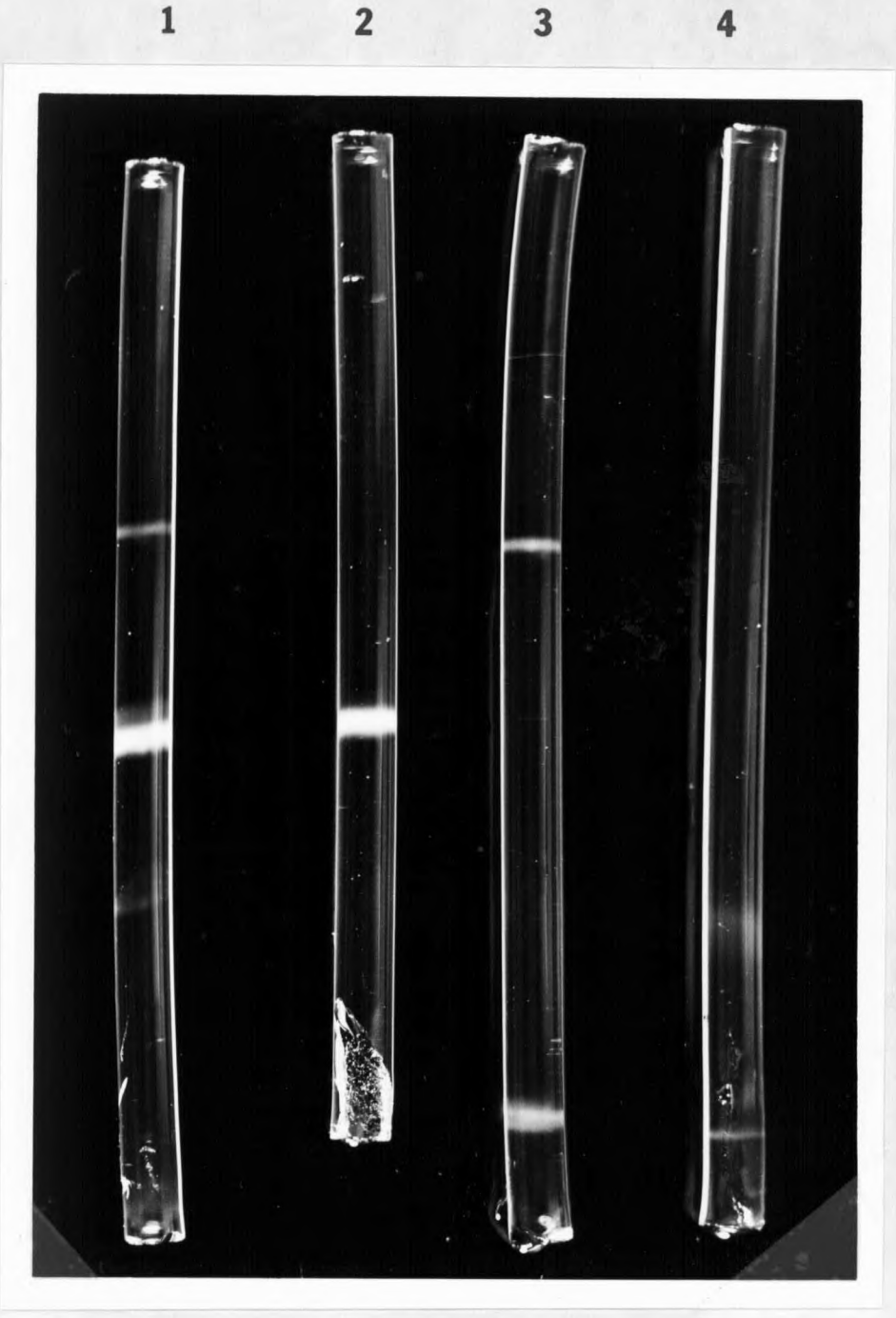

Figure 3. Isoelectric focusing of crude and purified sainfoin trypsin inhibitors. Gel 1, crude inhibitor; gel 2, inhibitor II; gel 3, inhibitor III; gel 4, inhibitor I.

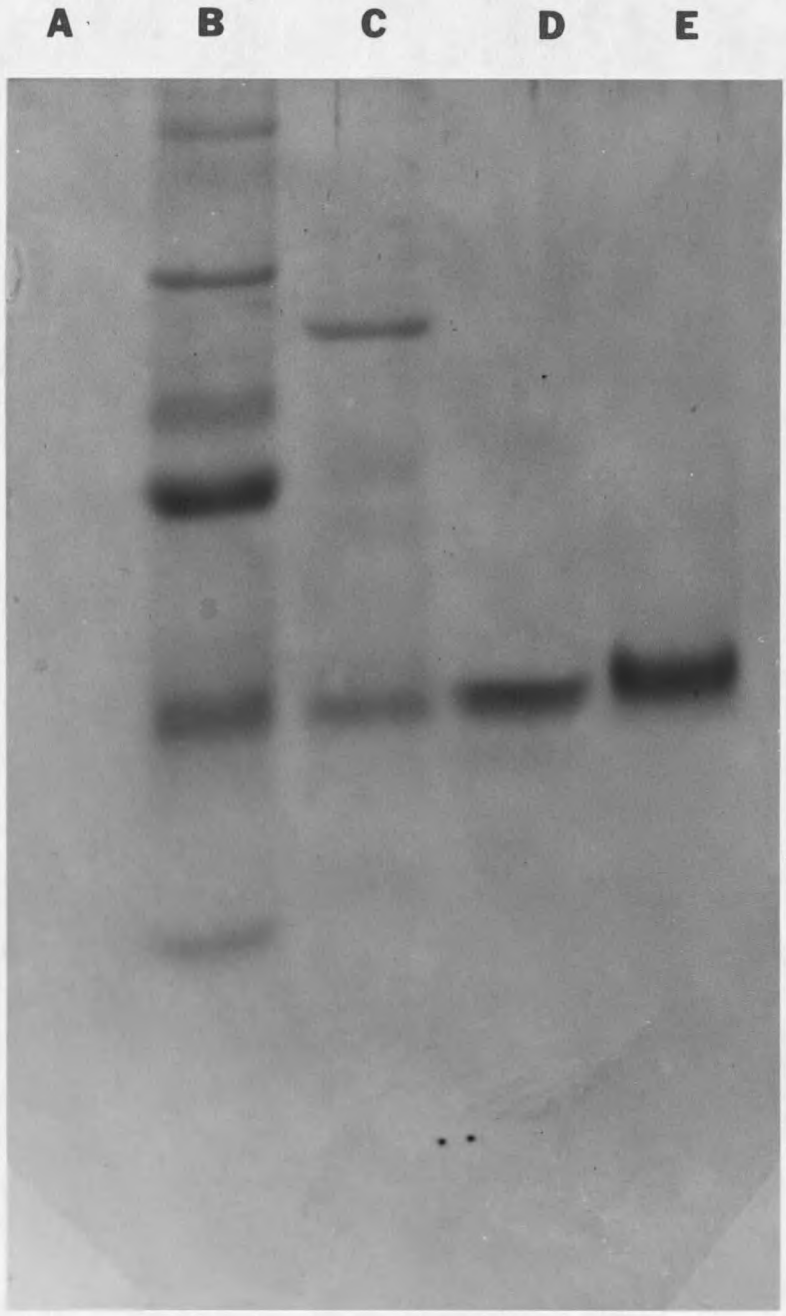

Figure 4. Urea SDS-poylacrylamide gel electrophoresis of crude sainfoin inhibitor and sainfoin inhibitor II. Lane A, control; lane B, molecular weight markers; lane C, crude sainfoin inhibitor; lane D, sainfoin inhibitor II; lane E, bovine pancreatic trypsin inhibitor.