ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF A TRYPSIN INHIBITOR FROM CORN (*ZEA MAYS L.*) SEEDS

by

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Introduction

In the plant kingdom certain storage organs such as seeds from the Leguminosae and Graminae families and tubers from the Solanaceae family are excellent sources of proteinase inhibitors. The inhibitors are quite diverse in number and in specificity towards various proteolytic enzymes. Sometimes several different kinds of inhibitors are present in a single tissue. For example, in soybeans (10, potato tubers (2), and lima beans (3), the inhibitors are present in multiple forms.

There has been much speculation concerning the physiological significance of plant proteinase inhibitors. Several explanations for their existence have been offered (1,4,5,6) but definitive experimental evidence is scarce, primarily because of the absence of suitable techniques to study the individual inhibitors within the tissues. Not only has the function of the inhibitors gone unproven, but little is known of the processes within the plants that control the existence of any proteinase inhibitor at a given time.

In 1967 Hochstrasser et al (7) reported an antitryptic activity in cornmeal (Zea mays). Halim (8) and Mertz (9) have shown that corn kernels containing the opaque-2 gene contain more trypsin inhibitor than corn kernels containing the floury-2 gene or normal gene. The research for the isolation and purification of trypsin inhibitor from opaque-2 corn kernels was undertaken with the conviction that one must understand thoroughly the chemistry of the inhibitor molecule if one is to fully understand its function at the molecular level within the plant tissue. In the following pages I will describe what we have learned of the purification and properties of a trypsin inhibitor of opaque-2 corn kernels.

Review of Literature

There are several recent reviews of the nutritional, pharmacological, and general aspects of proteinase inhibitors. Numerous references on proteinase inhibitors were cited in the reviews by Vogel et al (1) and Liener (5). Feeney and Allison (10) also tabulated the properties of many inhibitors in their review. "Methods in Enzymology" (11) contains a section on trypsin inhibitors. Ryan (6) has reviewed proteolytic enzymes and their inhibitors in plants. The Proceedings of the First and Second International Conferences on Proteinase Inhibitors are available in English and describe recent proteinase inhibitor research. Discussion of many topics not included herin may be found in these sources.

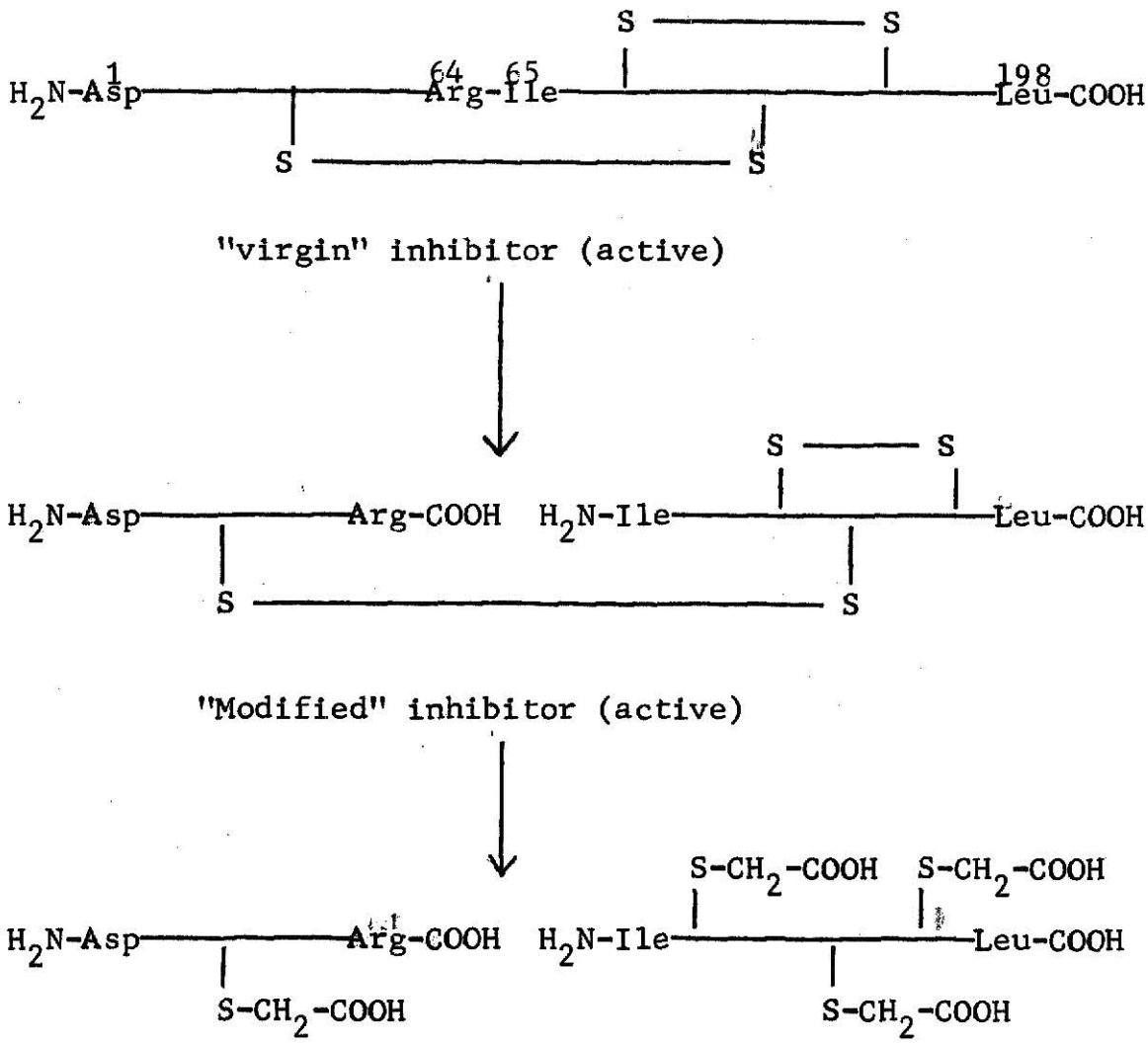
Probably the first plant trypsin inhibitor was that from soybean seeds, which was discovered in 1944 (12). The fundamental studies on the isolation and properties of this inhibitor were carried out by Kunitz, who succeeded in crystallizing the inhibitor (13) as well as the trypsin-trypsin inhibitor complex (14,15,16,17). Since its isolation and crystallization, soybean trypsin inhibitor has become the most extensively studied of the natural proteinase inhibitors, and has been used by many investigators as a model substance for investigation of the kinetics and thermodynamics of the inhibition of trypsin and of the properties of polypeptides in general. There are two important reasons for this: First, the inhibitor became available in the crystalline form soon after its discovery and has been marketed since as a salt-free product. Second, six percent of whole soybean protein is inhibitor protein.

The occurrence of proteinase inhibitors in plant organs such as seeds and tubers now is known to be widespread (1). They have been isolated mainly from the Leguminosae, Graminae, and Solanaceae families, but have been found in a number of other families (1,5,18). The plant proteinase inhibitors are generally small proteins having molecular weights under 50,000 and more commonly under 20,000 (1,18). Inhibitors from potatoes (6,19,20,21), corn (22), and several legumes (23-26) have monomer molecular weights below 10,000 and are often present as dimers and tetramers. Nearly all the plant inhibitors inhibit proteinases of animal or microbial origin having either trypsin-like and/or chymotrypsin-like specificities (1,5). A few inhibitors are known to inhibit endogenous proteinases of the plant from which they are derives (27,28,29), but in most instances inhibition of plant proteinases has not been reported.

Data on the chemical characteristics of pure, homogeneous proteinase inhibitors isolated from plants are limited. All apparently contain 9-20 percent cysteine in disulfide linkage (1). This extensive cross linking is considered to be an important factor for the stability of these inhibitors to denaturing conditions of heat, acid, alkali, and urea (18). All plant proteinase inhibitors so far examined contain proline, and many do not contain tryptophan (18,30). Reactive sites are thought to have unusual conformations and prolyl residues are frequently found or are postulated in those regions of proteins containing unusual conformational features. Hence, some investigators have proposed that the prolyl residues contribute to the required rigidity of the reactive sites of proteinase inhibitors (18). Some of the inhibitors are reported to contain a carbohydrate component while others are reported to be devoid of carbohydrate (5).

The inhibitors contain "active sites" for the inhibition of proteolytic enzymes that apparently endow them with their specificity (10,18). Laskowski et al (31,32,33) have contributed significantly to the understanding of the mechanism of interaction of trypsin inhibitors and trypsin. They observed that the interaction of trypsin and the Kunitz inhibitor involved the specific cleavage of an arginyl-isoleucine bond within the disulfide loop of the Kunitz inhibitor. The modified inhibitor was still active, but much more slowly than "virgin" inhibitor. However, removal of the newly formed C-terminal residue by treatment with carboxypeptidase B produced an inactive derivative. When the modified inhibitor was reduced and carboxymethylated, two fragments were formed, one having 64 residues and the other 134. (See diagram 1). On the basis of their results with soybean trypsin inhibitor (Kunitz) and with chicken ovomucoid, Laskowski et al (32) postulated that some trypsin inhibitors have a trypsin susceptible Arg-X bond in their reactive site while others have a Lys-X bond. These sites are part of a very large binding area that is necessary for the proper structural alignment that confers the unusual stability to the enzyme-inhibitor complex (34). However, the mechanism of inhibition has not yet been satisfactorily explained. To account for the stability of trypsin-trypsin inhibitor complexes, Laskowski et al (18) postulated the existence of a stable acyl intermediate between the enzyme and inhibitor. Evidence against the acyl-intermediate complex came first from studies of catalytically inactive enzyme derivatives (35-38) and more recently from X-ray diffraction studies of trypsin-trypsin inhibitor complexes (39,40).

Diagram 1

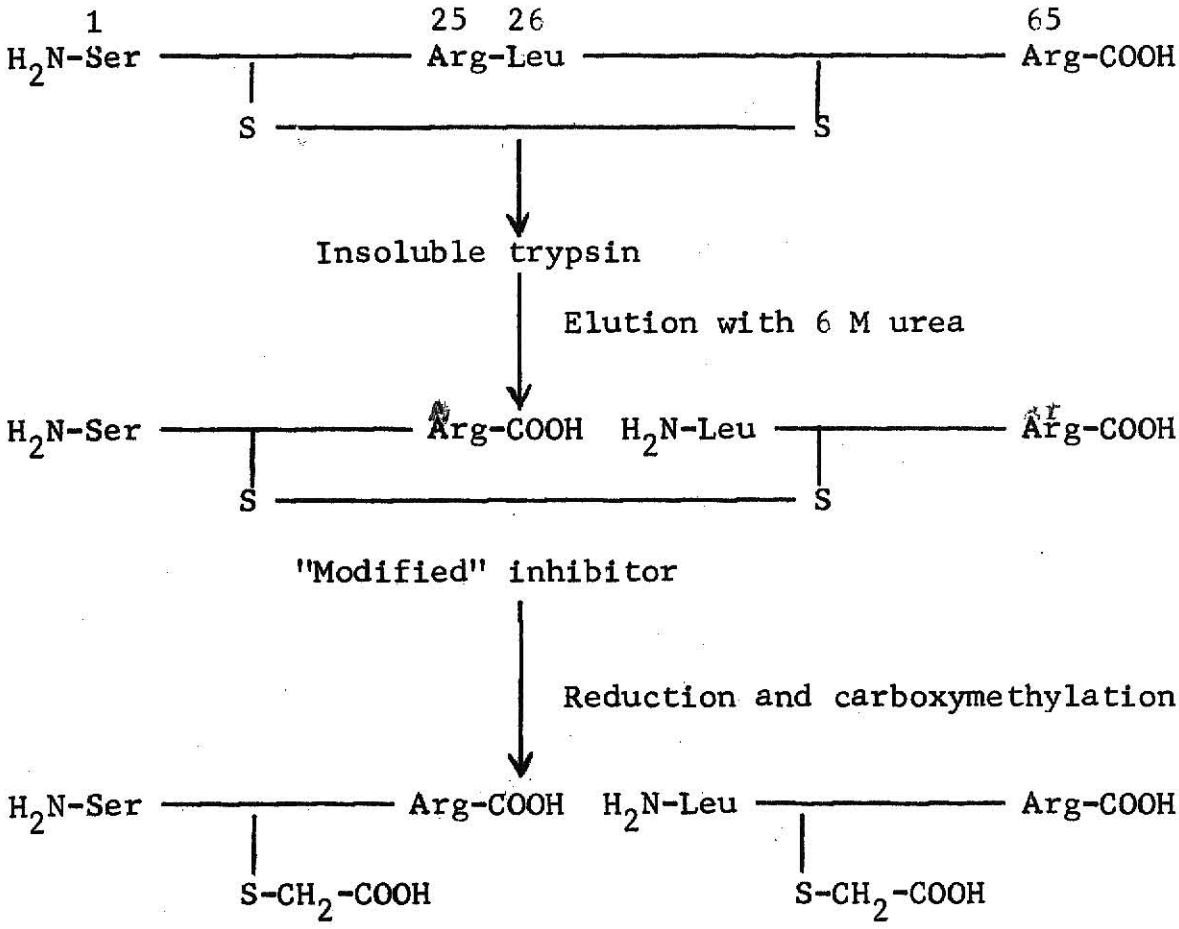


Two inactive fragments

In 1967 Hochstrasser et al (7) isolated a trypsin inhibitor from extracts of corn by taking advantage of an insoluble trypsin resin which forms a complex with this inhibitor. The inhibitor then could be dissociated from the insoluble complex by elution with 6M urea. The inhibitor was purified further by chromatography on CM-cellulose and Sephadex G-75. The inhibitor isolated by this procedure was presumed to have been modified by virtue of the trypsin cleavage (see diagram 2). The isolated inhibitor molecule consisted of two peptide chains which were connected to one another by S-S bridges. After reduction of the inhibitor, two fractions (not having inhibitor activity) were isolated by gel filtration. The modified inhibitor molecule was a carbohydrate-free protein. The molecular weight from Sephadex G-75 was 19,500. The same molecular weight was obtained from ultracentrifuge studies (22).

In 1970 Hochstrasser et al sequenced the trypsin inhibitor of maize seeds. The total number of amino acids found was only one third of the total amino acids calculated from the molecular weight and amino acid composition. Hochstrasser (22) therefore concluded the native trypsin inhibitor of corn was a polymer with subunits consisting of 65 amino acid residues.

Diagram 2



Two inactive fragments

Experimental

Trypsin Inhibitor Assay

The assay is based on a procedure developed by Erlanger (41) and uses N-benzoyl-DL-arginine-p-nitroanilide (BAPA) as the substrate for trypsin. The activity of trypsin is estimated by determining the rate of liberation of p-nitroaniline from BAPA by absorbance at 410 nm.

N_Benzoyl-D-argine-p-nitroaniline $\xrightarrow{\text{trypsin}}$ N-Benzoyl-D-arginine + p-nitroaniline.

Substrate stock solution: Dissolve 43.5 mg of DL-BAPA completely in one ml of dimethyl sulfoxide.
Enzyme Solution: Dissolve 10 mg of trypsin (Lyophilized trypsin, Worthington Biochemical Corporation, 242.9 U/mg) in 25 ml 0.001 M HCl.
Buffer Solution: (0.5 M Tris·Cl (trishydroxymethylaminomethane), pH 8.2, 0.02 M $CaCl_2$) Dissolve 60.57 g Tris and 2.94 g $CaCl_2 \cdot H_2O$ in 800 ml of distilled water, adjust to pH 8.2 with HCl, and dilute to one l with distilled water.
Inhibitor Solution: The inhibitor samples were prepared by diluting with 0.5 M tris, pH 8.2, containing 0.02 M $CaCl_2$.
Assay System: All solutions were pre-incubated at 25°C for ten minutes. Two assays were run to determine trypsin inhibition. First, the trypsin reference assay: Pipette into a 13 X 100 mm test tube 0.050 ml of trypsin solution and 0.225 ml of buffer solution. Mix and incubate at 25°C for ten minutes. To the incubation mixture add 1.7 ml of buffer solution, mix, and then pour into cuvette. Add 0.025 ml of substrate solution into the cuvette, mix, and follow

change in absorbance at 410 nm with a recording spectrophotometer. Second, the inhibitor assay: Pipette into a 13 X 100 mm test tube 0.050 ml of trypsin solution, 0.175 ml of buffer solution and 0.050 ml of inhibitor solution. Mix and incubate at 25°C for ten minutes. The rest of the procedure is the same as for the trypsin reference assay.

Direct estimation of enzyme activity was possible by comparison of the slopes of the linear portions of the plotted curves. Percent inhibition was determined by comparing enzyme activity without inhibitor to the enzyme activity with inhibitor. From Fig. 1 it can be seen that corn trypsin inhibitor activity deviates from linearity at higher inhibitor concentrations. Such deviation from linearity has been attributed by others to partial dissociation of the trypsin-inhibitor complex (42). Because of this deviation from linearity at high inhibitor concentrations, the inhibitor unit is defined as that amount of inhibitor which gives 50% inhibition under the above assay conditions.

Isolation and Purification of Trypsin Inhibitor from Corn Seeds

Preparative Procedure

Opaque-2 (1974) corn seeds (412 g) were ground as fine as possible with a burr mill. The ground corn was defatted by stirring for thirty minutes with one volume of acetone and removing by filtering under vacuum. This was repeated three times. The residue then was air dried. The residue was stirred for 5 hours at 4°C with 660 ml of 0.2 M NaCl. The extract was removed by filtering under vacuum at 4°C. The residue was re-extracted with 240 ml of 0.2 M NaCl for 6 hours. The two extracts were combined and centrifuged at 8,000 g for twenty minutes. The supernatant was adjusted to 0.05 M

Tris·Cl, pH 8.2, 0.02 M $CaCl_2$ and 1 M NaCl. This solution was centrifuged at 8,000 g for twenty minutes. The supernatant then was subjected to affinity chromatography.

Affinity Chromatography

Affinity chromatography was introduced as a method of enzyme purification by Cuatrecases et al (43). In contrast to other chromatographic techniques, which separate proteins on the basis of molecular weight, charge distributions, or both, affinity chromatography relies on selective biological interactions. Examples of such interactions are those between enzyme and substrate, enzyme and inhibitor, and antigen and antibody. Laeffer and Pierce (44) bound the enzyme trypsin to beaded agarose. The enzymatically active immobilized trypsin has a specific and high binding capacity for the naturally occurring trypsin inhibitor of soybean. It was thought that this technique might be used for the isolation of the trypsin inhibitor of corn.

One hundred ml of Sepharose 4B was activated according to the procedure of Cuatrecases et al (43). Sepharose was placed in a sintered glass funnel and gently stirred during the activation by bubbling nitrogen gas up through the funnel. The pH during the activation was maintained at 11.0 by the addition of 5 N NaOH. One gram of trypsin was reacted with the activated Sepharose, using 0.02 M borate buffer, pH 9.0 containing 0.1 M $CaCl_2$ according to the procedure of Laeffler and Pierce (44). The mixture was shaken gently at 4°C for 24 hours. Only about 40 percent of the enzyme could be removed by extensive washing of the polymer with the borate buffer. Therefore, only 60 percent of the trypsin was coupled to the Sepharose. The Sepharose was then reacted

Fig. 1. Inhibition of trypsin activity as a function of the level of crude corn extract, using BAPA as substrate. Dashed line is extrapolated from the linear portion of the curve.

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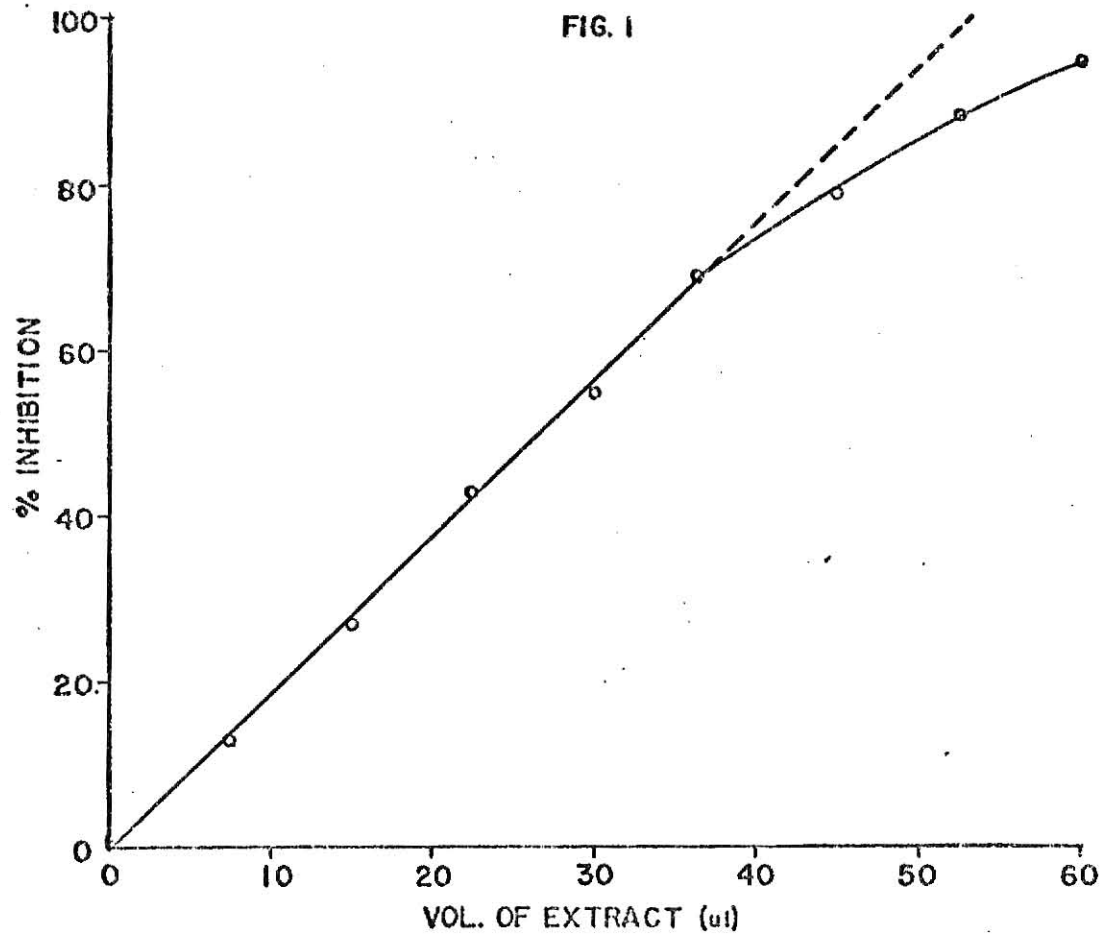
FIG. 1



at 4°C with 100 ml of a 1 M 2-aminoethanol for 12 hours, as a precautionary measure, to couple any remaining active Sepharose sites.

For isolation of trypsin from corn, a 2.6 X 18.5 cm column of Trypsin-Sepharose 4B with 0.6 g of trypsin attached was used at a flow rate of 140 ml/hr. The column was equilibrated with 0.05 M Tris·Cl buffer, pH 8.2, containing 0.02 M $CaCl_2$ and 1 M NaCl. The high concentration of NaCl was used to minimize possible nonspecific or electrostatic binding of molecules in the corn extract to the column. The supernatant from the preparative procedure was applied to the column and 20 ml fractions of the effluent were collected. After all the supernatant had been applied to the column, the starting buffer (0.05 M Tris, pH 8.2, 0.02 M $CaCl_2$ and 1 M NaCl) again was applied to the column to elute any protein not specifically adsorbed.

After the absorbance at 280 nm of the effluent from the trypsin-Sepharose 4B column had decreased to less than 0.025 a solution composed of 1 M acetic acid and 0.02 M $CaCl_2$ (44) was applied to the column. The trypsin inhibitor activity then appeared in the effluent (Fig. 3).

From Fig. 3 it can be seen that no trypsin inhibitor activity was detected in the effluent from the trypsin-Sepharose column until the column was washed with the 1 M acetic acid and 0.02 M $CaCl_2$ solution. The peak appearing in the effluent after the application of the latter solution contained 94 percent of the trypsin inhibitor activity applied to the column.

Polyacrylamide Disc Gel Electrophoresis

The active peak from the trypsin-Sepharose 4B column was examined for homogenity by analytical polyacrylamide disc gel electrophoresis, using the procedure of David (45). The reservoir buffer was prepared with distilled at 4°C, ice was packed around the buffer reservoirs, and the electrophoresis was performed at room temperature. Gels were stained with one percent amide black in 7 percent acetic acid and 40 percent ethanol, for thirty minutes. They were destained in 7 percent acetic acid and 40 percent ethanol with the aid of Dowex-1 anion exchange resin.

Preliminary experiments with gels of varying percentages of acrylamide showed that gels of 7.5 percent acrylamide gave the best resolution. Therefore, the inhibitor fraction was subjected to 7.5 percent acrylamide gel electrophoresis at pH 4.3 and 8.3. Electrophoresis at pH 4.3 resulted in a single band. However, electrophoresis at pH 8.3 resulted in two major bands and one very minor band (Fig. 4). These results indicate that the active peak from the trypsin-Sepharose 4B column was heterogeneous.

Preliminary Chromatography

Robinson et al (46) had shown that α and β trypsin bound to a chicken ovomucoid affinity column were selectively eluted by a pH gradient. All attempts to elute the corn trypsin inhibitor from the affinity column by using pH gradients failed to resolve the bands observed on gel electrophoresis. Chromatography with Sephadex G-100 at various pH's did not separate the bands. Experiments employing carboxymethyl (CM) cellulose also failed to resolve the inhibitor bands. However, preliminary experiments with diethylaminoethyl

Fig. 2. Chromatography of corn trypsin inhibitor on Sepharose 4B. (Column was run at 4°C.)

Fig. 3. Inhibitor units in fractions from the Sepharose 4B column.

FIG. 2

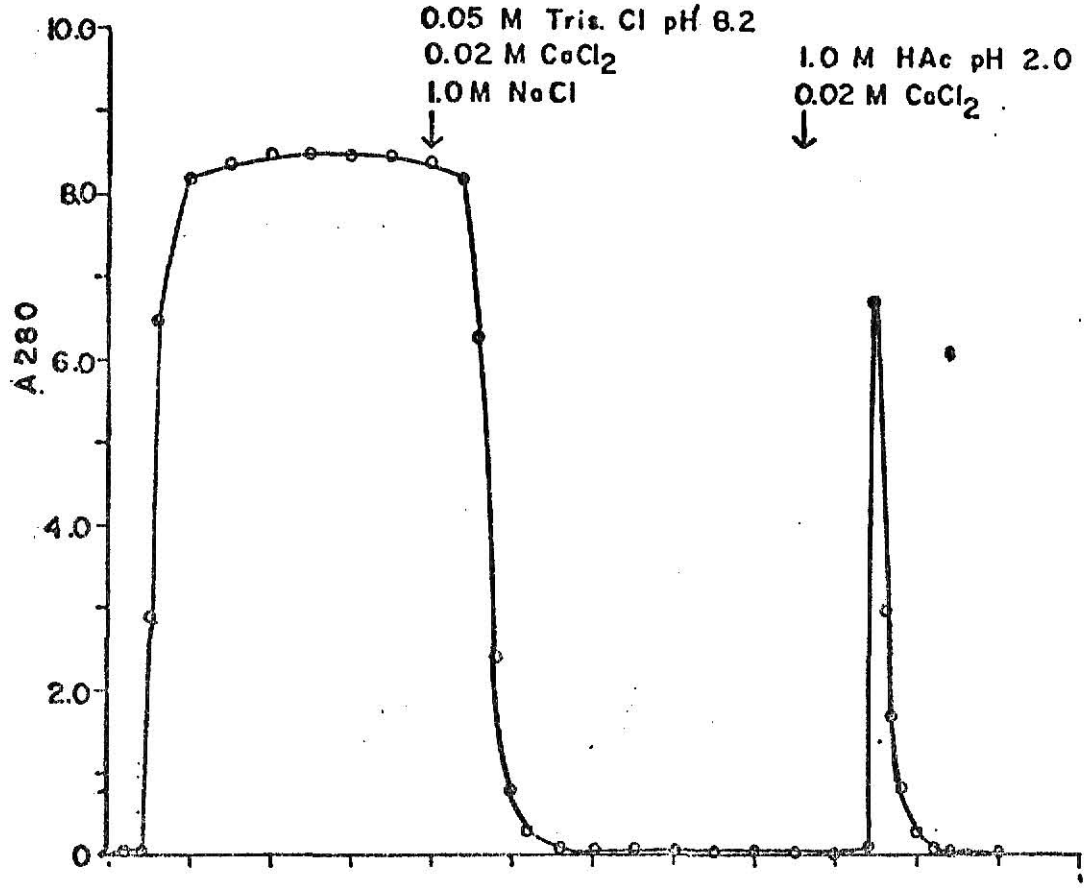
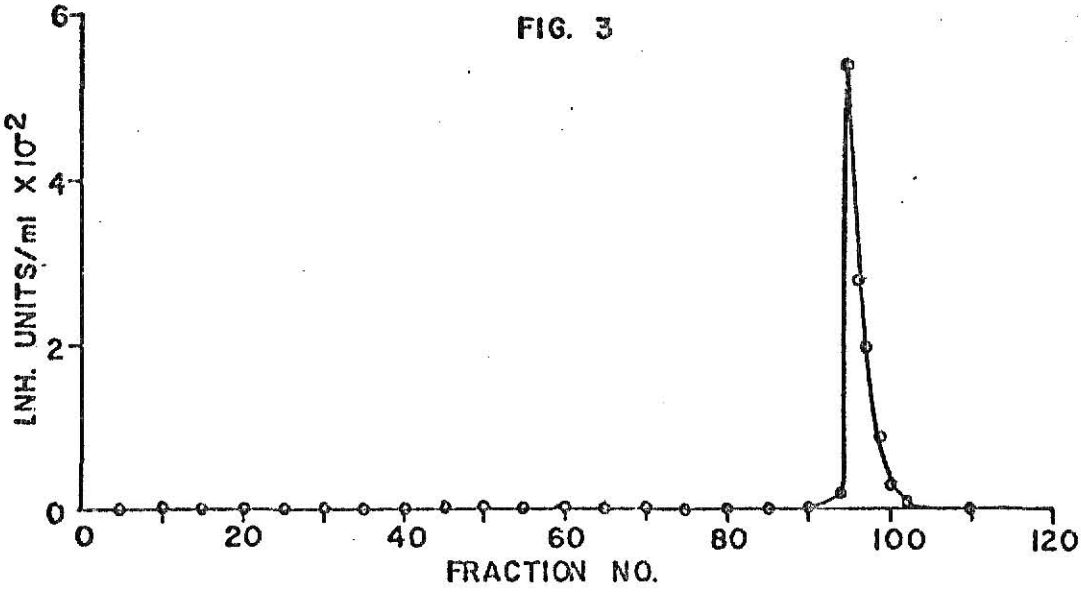

0.05 M Tris. Cl pH 8.2
0.02 M $CaCl_2$
1.0 M NaCl
1.0 M HAc pH 2.0
0.02 M $CaCl_2$
A 280
10.0
8.0
6.0
4.0
2.0
0

FIG. 3


INH. UNITS/ml X 10^{-2}
6
4
2
0
0
20
40
60
80
100
120
FRACTION NO.

(DEAE) cellulose resolved at least the slower moving band from the other two bands observed on gel electrophoresis.

DEAE Cellulose Chromatography

Forty g (dry wt.) of DE-32 was prepared according to the Whatman leaflet (47). The active effluents from the trypsin-Sepharose 4B column were combined (150 ml) and dialyzed against 8 liters of 0.01 M 2-amino-2-methyl-propanediol buffer, pH 9.0, for 6 hours. The buffer solution was then changed and the dialysis continued another 6 hours. The dialyzed sample was made 8 M with urea and applied at 40 ml/hr to a 1.6 X 80 cm column of DE-32 cellulose which had been equilibrated with 0.01 M 2-amino-2-methyl-propanediol buffer, pH 9.0, containing 8 M urea. The column then was washed with 200 ml of the equilibrating buffer. The inhibitor was eluted by means of a linear salt gradient varying from 0.0 to 0.1 M NaCl in a total volume of 1,000 ml of 0.01 M 2-amino-2-methyl-propanediol buffer, pH 9.0, containing 8 M urea. The elution profile and inhibitor assays are shown in Fig. 5. It can be seen that the trypsin inhibitor activity follows the protein elution profile (determined by absorbances at 280 nm). Three fractions designated A, B, and C were concentrated by the following procedure. They were dialyzed against 8 liters of 2-amino-2-methyl-propanediol buffer, pH 9.0, for 5 hours. The fractions then were again applied at 70 ml/hr to 1.6 X 23 cm DEAE cellulose columns equilibrated with the same buffer. The columns were washed extensively with the above buffer to remove urea. The fractions from the DEAE column were exposed to urea no longer than thirty-six hours. The urea was removed as soon as possible to minimize the possible reaction of cyanate with nucleophilic groups in proteins

Fig. 4. Polyacrylamide gel electrophoresis at pH 8.3. Migration was from top to bottom. From left to right: Sepharose 4B active fraction, fraction A, B, and C from DEAE column.

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Fig. 4

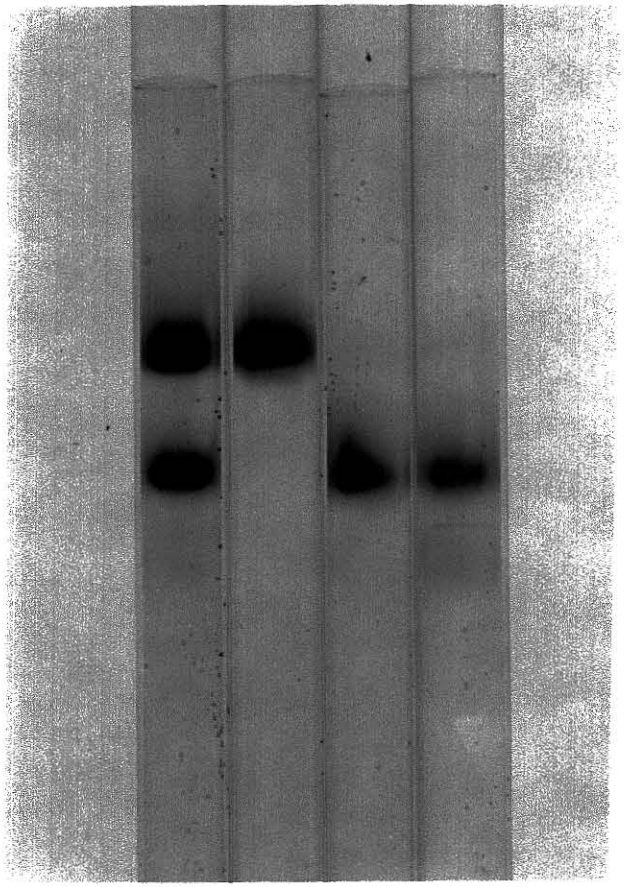

Fig. 5. DEAE chromatography of the active fraction from Sepharose 4B. The column was operated at 4°C. Five ml fractions were collected.

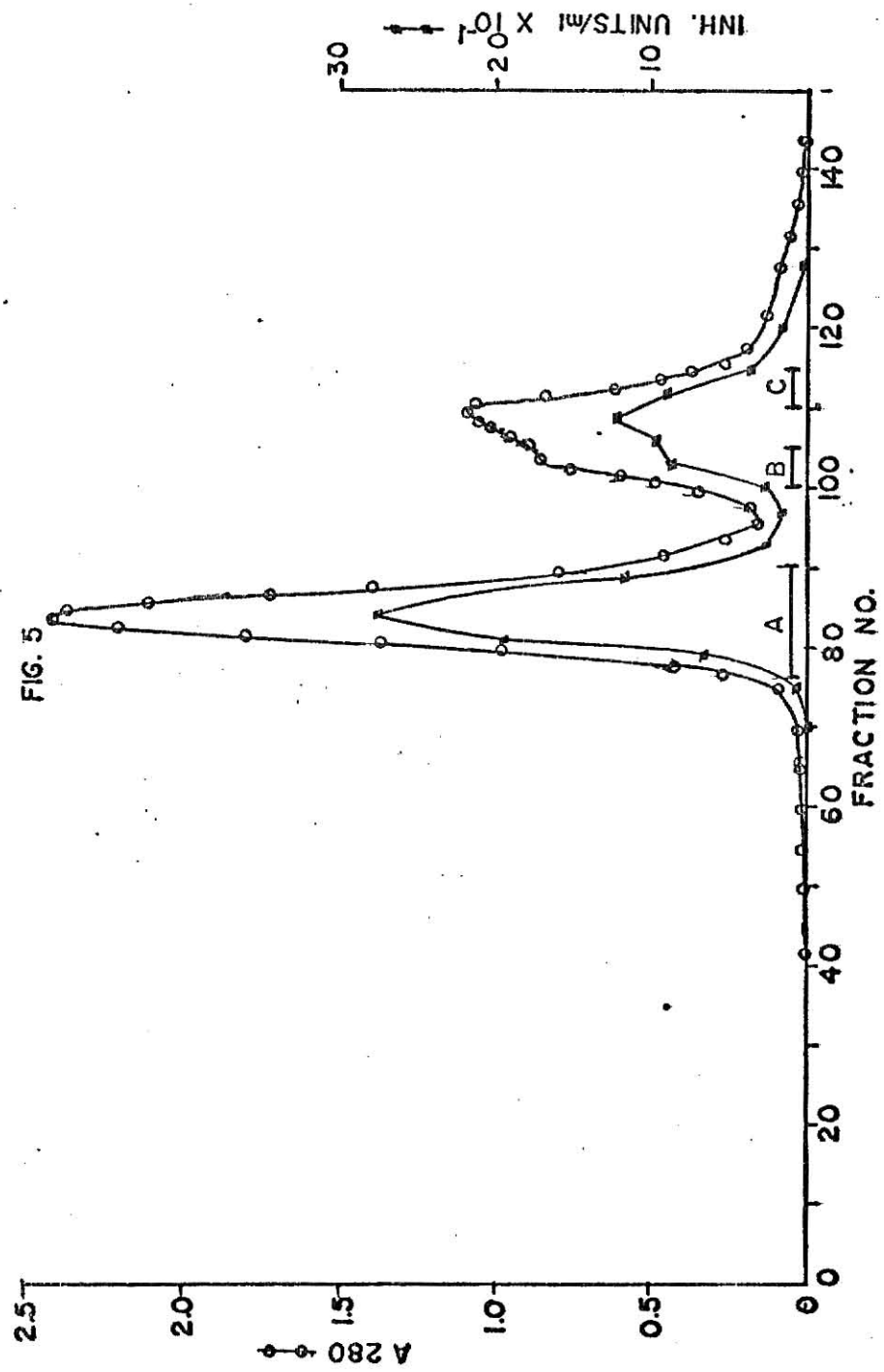

FIG. 5

to yield carbamyl derivatives. The trypsin inhibitor fractions then were eluted from the DEAE cellulose columns with 0.2 M NaCl.

Aliquots of fractions A, B, and C were subjected to 7.5 percent acrylamide disc gel electrophoresis at pH 8.3. The results are shown in Fig. 4. The gel for fraction A showed one band, identical to band one of the Sepharose 4B gel. The gel for fraction B showed two faint bands and one dark band. These bands have mobilities identical to those of the Sepharose 4B bands. The majority of the material in fraction B was identical to band 2 of the Sepharose 4B gel. Fraction C is quite similar to that of fraction B.

A summary of the purification of trypsin inhibitor from corn is presented in Table 1.

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Table 1. Purification of Corn Trypsin Inhibitor

Step	Volume	Inhibitor units (a)	Total Inhibitor units (b)	Protein	Specific activity (c)	Yield (d)	Relative purification (e)
	ml	ml^{-1}		mg ml^{-1}	mg^{-1}	%	
Crude Extract (0.2 M NaCl)	730	37	26,718	1.6[f]	23	100	1.0
Tris pH 8.2 + 0.2 M $CaCl_2$ 1 M NaCl--after centrifugation	740	35	25,900	1.2[f]	29	97	1.3
Sepharose 4B active fraction	153	156	23,868	0.7[f]	223	89	9.7
Trypsin inhibitor from DEAE column	228	85	19,298	---	---	72	---
Concentrated fractions from DEAE column[h]							
A	68	127	8,636	0.55[g]	230	32	10
B	24	80	1,920	0.35[g]	228	7	9.9
C	24	51	1,224	0.22[g]	231	5	10

See the next page for explanation of footnotes.

(a) Inhibitor unit is the amount of inhibitor in ml that gives 50 percent inhibition under the defined assay conditions.

(b) Total inhibitor units were calculated as: Volume X Inhibitor units.

(c) Specific activity was calculated by: Inhibitor units/ Protein

(d) Yield calculated by: Total Inhibitor units / Total inhibitor units in the crude extract.

(e) Relative Purification calculated by: Specific activity/ Specific activity of crude extract

(f) Protein concentration determined by Lowry method (68)

(g) Protein concentration determined at 280 nm using $E_{1\ cm}^{1\%} = 2.0$.

(h) Fractions A, B, and C do not represent all the inhibitor activity from the DEAE column (Fig. 5).

CHARACTERIZATION OF THE TRYPSIN INHIBITOR ISOLATED FROM CORN

Sodium Dodecyl Sulfate Disc Gel Electrophoresis

The general procedure of Weber and Osborn (50) was used, with the following modifications: The amount of bromphenol blue recommended for the above procedure became so diffuse after running the gels for four hours that it was impossible to determine the location of the marker dye in the gel. Therefore the quantity of dye was increased from 3 ul to 40 ul. Staining was accomplished by exposing the gels to 1 percent amido black in 7 percent acetic acid and 40 percent ethanol for 30 minutes. Destaining was accomplished with 7 percent acetic acid, 40 percent ethanol and Dowex-1 anion exchange resin. After electrophoresis, the dye front was marked by cutting off the gel below the dye front with a razor blade. Mobilities were calculated after destaining by dividing the distance which the protein band had migrated by the length of the gel.

Aliquots of fractions A, B, C, and of the active material from the Sepharose 4B column were subjected to 15 percent polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate and B-mercaptoethanol. The gels were 10 cm long. As seen from Fig. 6, the gel for fraction A showed one band. The mobility of the fraction A band was identical to the mobility of band 1 seen on the gels of fractions B, C, and Sepharose 4B. The mobilities of the second and third bands on the gels of Band C are identical to the corresponding bands on the Sepharose 4B gel.

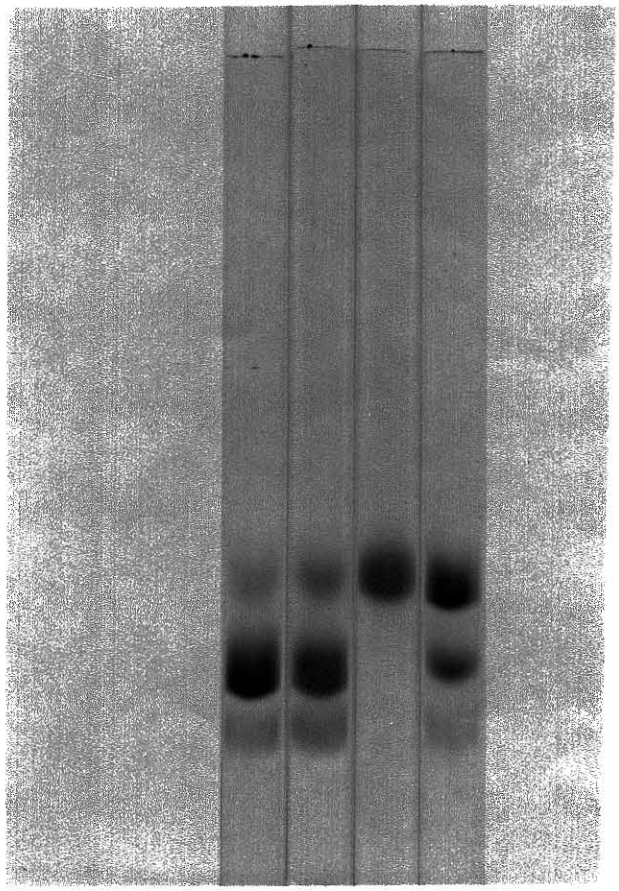
Weber and Osborn (50) found, after examining several standard proteins and plotting their mobilities against the

logarithms of their molecular weight (M.W.), that one obtains a straight line from which it is possible to estimate the M.W. of an unknown polypeptide within ± 10 percent. Weber and Osborn (50) used only proteins in the molecular weight range from 11,000 to 70,000. Polypeptides below 11,000 M.W. were not examined. Fig. 7 is a plot of molecular weight vs migration distance for several standard proteins using the system of Weber and Osborn (50), with the previously mentioned modifications. The lowest M.W. standard in Fig. 7 is that of bovine pancreatic secretory trypsin inhibitor (PTI), M.W. 6,161 (30). Bovine insulin was considered as a M.W. standard, but repeated experiments with a variety of reducing conditions failed to separate insulin into its α and B chains. Thus, bovine insulin was not included as a M.W. standard. Although only one standard point (PTI) is plotted in the low molecular weight region, there seems to be a break in the plot of standards at around 9,000 M.W. The validity of using a break, as seen in Fig. 7, can be supported by the work of Swank and Munkers. Swank and Munkers (51) found a negative linear correlation between log molecular weight and migration distance for peptides between 1,000 and 10,000 M.W., ± 18 percent. However, they found that when the linear correlations between log molecular weight and migration distance of large proteins (10,000-70,000 M.W.) were compared with those of small peptides (1,000-10,000 M.W.), a break in the plot occurs at around 10,000 to 12,000 M.W. Using the data of Fig. 6 and Fig. 7, band 1, or the top band on the S.D.S. gel, has a M.W. of approximately 4,000. The M.W.'s of band 2 and band 3 add up to 10,000, which is close to the M.W. of 10,500 for the first band.

In combination with the data from disc gels run without sodium dodecyl sulfate (Fig. 4), fraction A appears to contain a polypeptide with a single chain. The main band of fractions

Fig. 6. Sodium dodecyl sulfate gels. Migration is from top to bottom. From right to left: Sepharose 4B active fraction, fractions A, B, and C.

Fig. 6



B and C (Fig. 4), although contaminated with some of the material in fraction A, appears to be made of two polypeptide chains which appear as two bands. The combined M.W. of these two bands is close to the M.W. of the isolated band of fraction A.

Re-chromatography on the affinity column

An aliquot of fraction A from the DEAE cellulose chromatography was re-bound to the trypsin-Sepharose 4B column and the active fraction eluted with 1 M acetic acid containing 0.02 M $CaCl_2$. An aliquot of fraction A before binding to the affinity column showed a single band after non-SDS gel electrophoresis, identical to the one shown in Fig. 4. for fraction A. However, the active fraction collected after being re-bound to the affinity column showed two bands on non-SDS gel electrophoresis identical to bands 1 and 2 shown in Fig. 4 for the Sepharose 4B fraction. These data provide strong evidence that the two main bands observed on gel electrophoresis (Fig. 4) of the active fraction from the Sepharose 4B column do not represent two different trypsin inhibitors existing naturally in corn seeds, but rather one inhibitor which has been chemically modified by the trypsin of the affinity column. The mechanism for this modification may be similar to the one proposed by Laskowski (33) where "virgin" inhibitor is converted by the enzyme trypsin to a modified inhibitor which has had one peptide bond cleaved but remains intact due to disulfide bonding.

Carbohydrate Content

Tje presence of carbohydrate in fractions A, B, and C from the DEAE column were determined by the phenol-sulfuric acid reaction of carbohydrates (52). This is a rapid and reproducible procedure for the determination of simple sugars, oligosaccharides, polysaccharides and their derivatives, including the methyl esters with free or potentially free reducing groups. Such substances give an orange-yellow color when treated with phenol and concentrated sulfuric acid. The method is not affected by the presence of proteins, and thus it is a useful technique for estimating the carbohydrate content of glycoproteins. However, the amino sugars, such as glucosamine and galactosamine, are inert in this procedure.

A small amount of orange-yellow color developed in the phenol-sulfuric acid reaction with aliquots from fractions A, B, and C from the DEAE column. On comparing the absorbances at 490 nm to a standard curve of D-mannose, all three fractions (A,B,C) appear to contain 5 ug of carbohydrate/mg of protein[a].

Extinction Coefficient Determination

A solution of fraction A (A_{280}= 2.210) was equilibrated to 0.01 M phosphate buffer, pH 6.8, containing 0.1 M KCl and was centrifuged at 10,000 rpm in a Beckman model E analytical ultracentrifuge, using a synthetic boundary cell. The fringes were counted (4.54) across the protein boundary. Using a refractive increment of 4.10 (53), the protein concentration was calculated to be 1.108 mg/ml. Therefore, at 280 nm, $E^{1\%}_{1\ cm}$= 2.0.

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[a] Protein concentration was determined at 280 nm using $E^{1\%}_{1\ cm}$=2.0

Amino Acid Analysis

A Beckman 120 amino acid analyzer was used for all analysis. All amino acid compositions were derived from duplicate analyses of the protein samples. Fraction A was hydrolyzed in 6 N HCl at 110°C for 25, 36, 48, and 72 hours. Serine and threonine were determined by plotting values obtained from the amino acid analysis after 24, 36, 48, and 72 hr of hydrolysis versus time and the "true" value was obtained by extrapolating to zero time. Values for isoleucine and valine are the average values taken at 72 hours of hydrolysis. Half-cystine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation (54). Fraction C was hydrolyzed for 24 hours only. All individual amino acid analyses, except tryptophan, were converted to a common molar basis by adjusting all alanine values to an integral number. The spectrophotometric method of Edelhoch (55) was used to determine tryptophan. A Cary spectrophotometer Model 14 was employed to determine the absorbance spectrum of fraction A in 0.02 M phosphate, pH 6.7, containing 6.0 M guanidine hydrochloride. Its spectrum is shown in Fig. 8. The absorbance of half-cystine and tyrosine were subtracted out by using the contents shown in Table 2 and Edelhoch's molar extinction coefficients. The content of tryptophan was then determined using Edelhoch's extinction coefficient at 280 nm for N-acetyl-tryptophan amide and a molecular weight of 10,500 for the inhibitor molecule. The amino acid composition of fraction A is presented in Table 2. Of particular interest are the high contents of half-cytine and proline and the presence of tryptophan.

Table 3 contains the molar amino acid compositions of fractions A and C and of Hochstrasser's corn trypsin inhibitor. Table 3 also contains the amino acid composition of Hochstrasser's corn inhibitor based on his sequence data on the corn inhibitor (22). The similarities in fraction A, C, and Hochstrasser's amino acid composition are further evidence that these corn trypsin inhibitor samples are quite similar. However a marked difference can be noted between the amino acid composition based on Hochstrasser's sequence (22) and that of fraction A, C, and even his own amino acid composition in Table 2.

p-Mercuribenzoate (PMB)

The sulfhydryl groups of proteins will react with mercurials to form mercaptides. p-Mercuribenzoate reacts with free sulfhydryl groups of proteins to form monomercaptides. The formation of these mercaptides gives rise to spectral changes that can be followed analytically with a spectrophotometer (56). The procedure of Methods of Enzymology (57) was used to determine if free sulfhydryl groups exist in the corn trypsin inhibitor. Preliminary experiments with PMB showed that this technique was sensitive enough to detect free sulfhydryl groups at a concentration of 2.0×10^{-5} M. Fraction A (2.3×10^{-5} M in 0.33 M acetate buffer, pH 4.6, containing 8 M guanidine hydrochloride) was adjusted to 2.2×10^{-5} M PMB. No spectral change was observed even after letting the reaction mixture stand for twenty-four hours. This indicates there are no free sulfhydryl groups in the corn trypsin inhibitor of fraction A.

Fig. 7. Plot of molecular weight vs migration distance. Standard proteins were bovine serum albumin (BSA), 68,000 (50), alcohol dehydrogenase (ADH), 41,000 (50), trypsin (Try), 23,300 (50), soybean Trypsin Inhibitor (SBI), 20,000 (30), ribonuclease (Ribo), 13,700 (50), lima bean trypsin inhibitor (LBI), 9,195 (30), and pancreatic secretory trypsin inhibitor (PTI), 6,023 (30).

FIG. 7

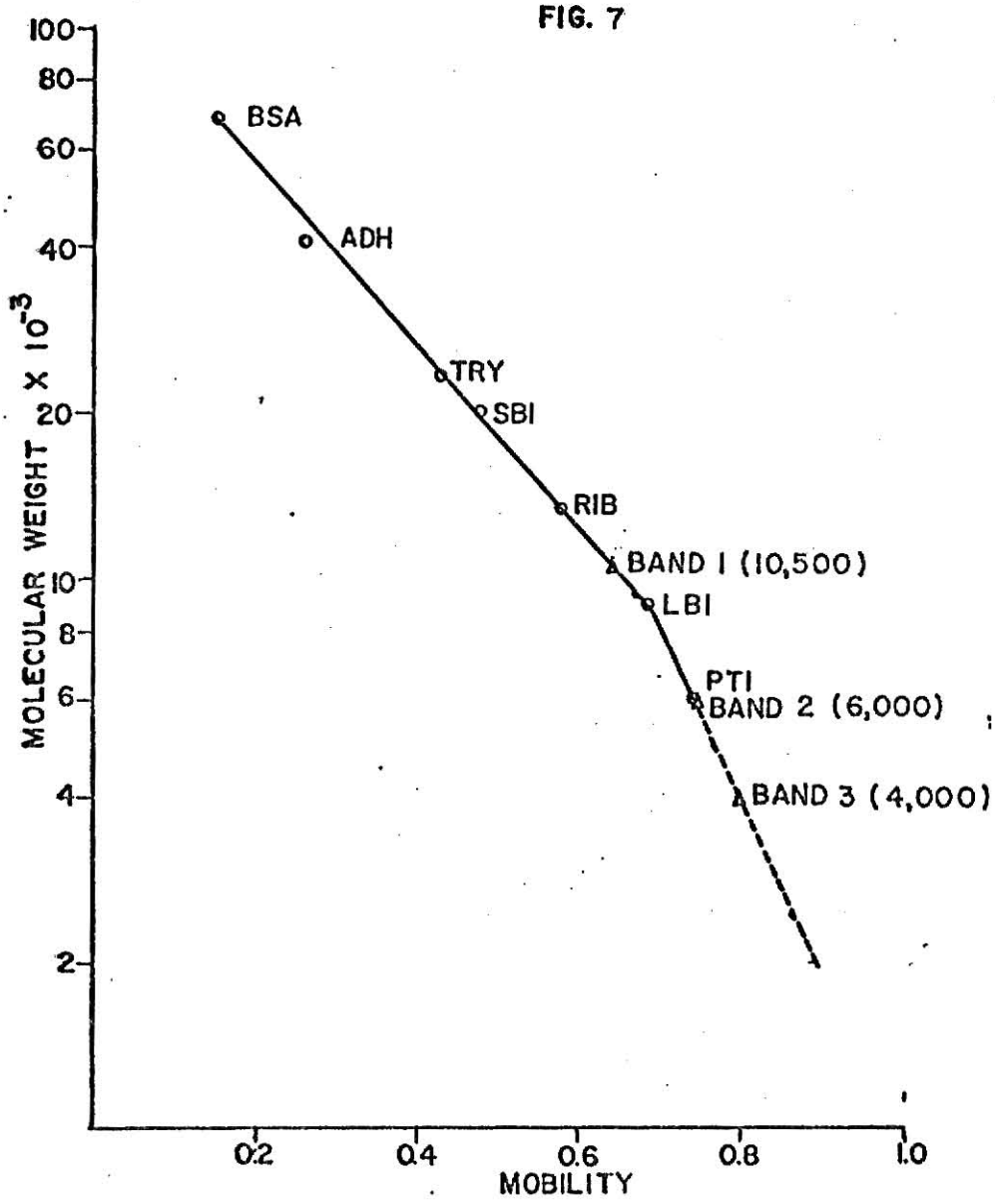

100
80
60
40
20
10
8
6
4
2
MOLECULAR WEIGHT X 10^{-3}
BSA
ADH
TRY
SBI
RIB
BAND 1 (10,500)
LBI
PTI
BAND 2 (6,000)
BAND 3 (4,000)
0.2
0.4
0.6
0.8
1.0
MOBILITY

Fig. 8. Absorbance spectrum of fraction A in 0.02 M phosphate, pH 6.7 containing 6.0 M guanidine hydrochloride.

FIG. 8

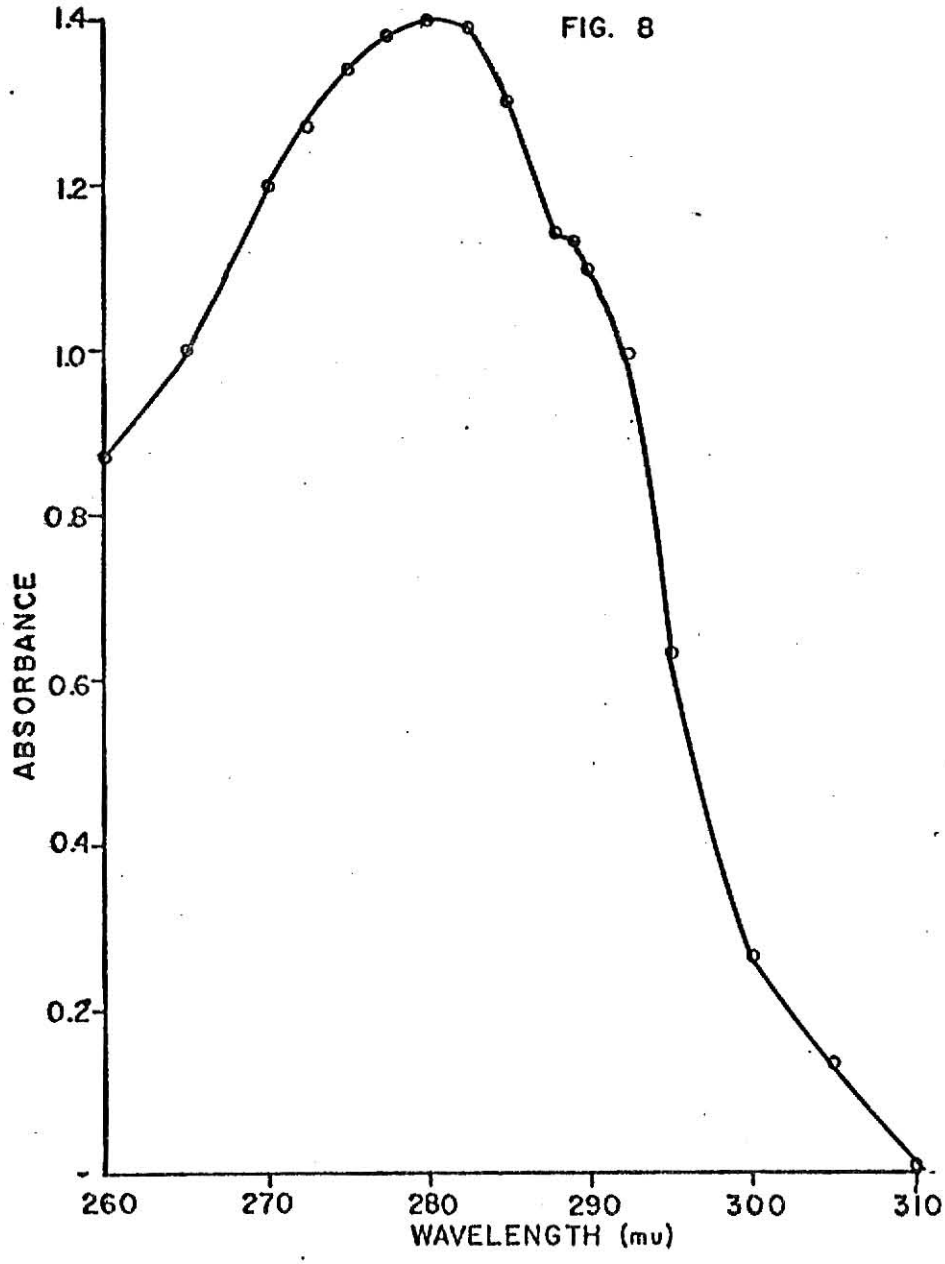

1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
260
270
280
290
300
310
WAVELENGTH (mu)

Table 2

Hydrolysis of Fraction A for varying lengths of time

Amino Acid	Amount formed after hydrolysis for[a]					Moles of amino acids based on a molecular weight of 10500
	24 hr	36 hr	48 hr	72 hr	average	
Lysine	1.91	1.93			1.92	0.95
Histidine	1.75	1.80			1.78	0.88
Arginine	15.80	16.18			15.99	7.88
Aspartic acid	10.04	10.27	10.52	10.35	10.30	5.08
Threonine	11.02	11.13	10.85	9.63	12.05	5.94
Serine	8.19	8.56	7.40	5.97	9.60	4.73
Glutamic acid	17.39	17.60	18.24	18.03	17.82	8.78
Proline	22.31	22.62	23.67	23.06	22.92	11.29
Glycine	21.09	22.88	21.75	21.85	21.89	10.78
Alanine	20.00	20.00	20.00	20.00	20.00	9.85
Valine	8.55	8.57	8.60	8.68	8.68	4.28
Isoleucine	11.23	11.08	11.13	11.29	11.29	5.56
Leucine	19.76	19.80	20.28	20.58	20.11	9.91
Tyrosine	2.87	2.87	3.00	2.83	2.87	1.41
Phenylalanine	1.85	1.90	1.81	1.95	1.88	0.93
Methionine					2.17	1.07
Half-cystine					13.03	6.4
Tryptophan						3.34

a) based on 20 moles of alanine

Table 3

Amino Acid	Fraction A	Fraction B	Hochstrasser's amino acid composition	Amino acid composition based on Hochstrasser's sequence
Lysine	0.95	1.10	1.06	1.46
Histidine	0.88	0.91	1.06	1.46
Arginine	7.88	7.89	7.86	11.72
Aspartic acid	5.08	5.39	5.52	4.39
Threonine	5.94	5.64	5.98	4.39
Serine	4.73	4.26	6.07	4.39
Glutamic acid	8.78	9.05	9.01	7.32
Proline	11.29	11.59	11.64	14.64
Glycine	10.78	10.44	9.93	10.25
Alanine	9.85	9.77	9.43	5.86
Valine	4.28	4.16	4.14	2.92
Isoleucine	5.56	5.68	5.52	5.86
Leucine	9.91	9.80	8.83	8.77
Tyrosine	1.41	1.42	1.52	1.46
Phenylalanine	0.93	0.80	1.05	0.00
Methionine	1.07	0.93	0.41	1.46

The composition of Fraction A is from Table 1. All other compositions were adjusted to the same number of daltons (9,286) as computed from Fraction A.

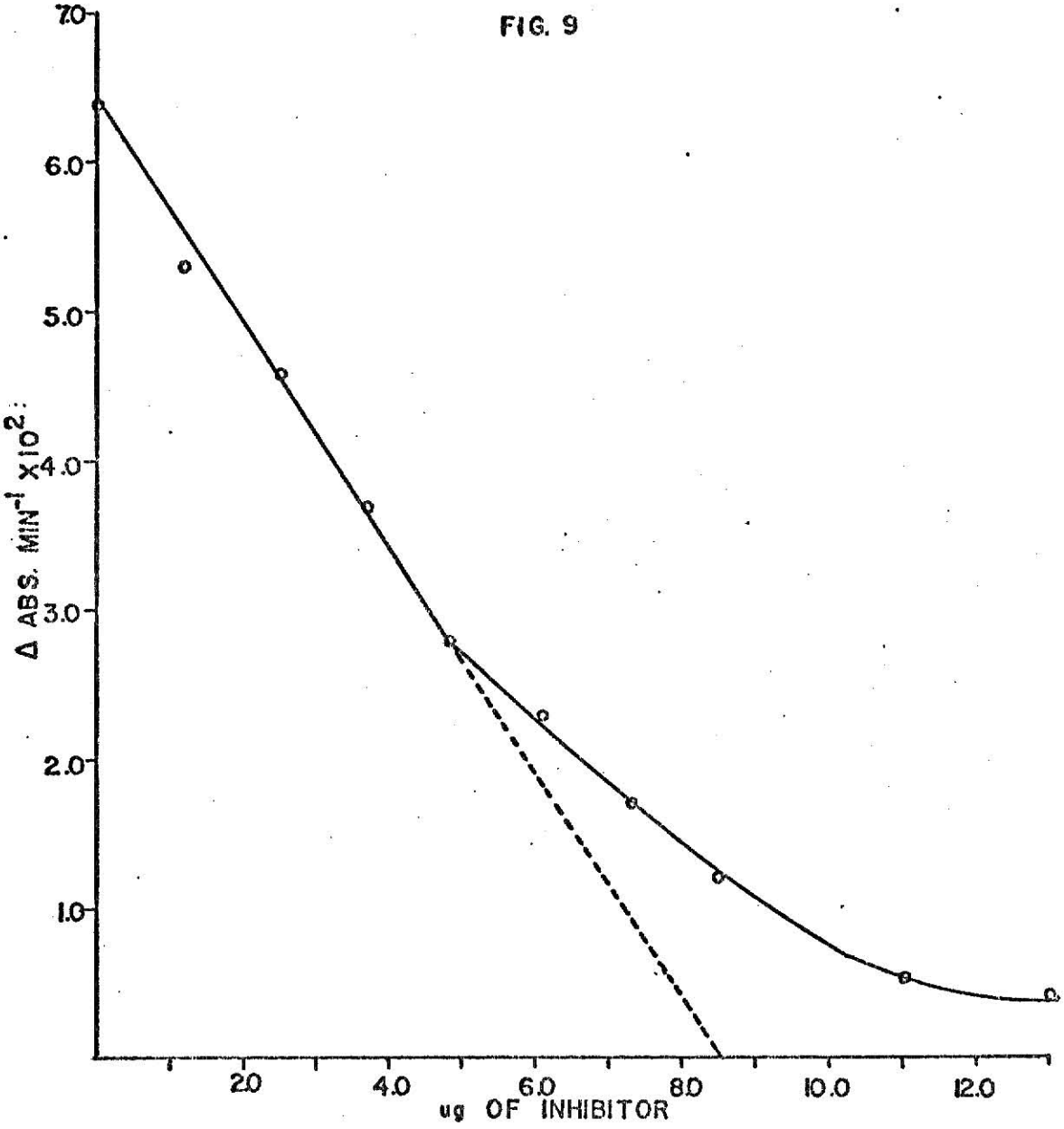
Determination of Stoichiometry

The assay for trypsin activity and the decrease in activity with the addition of inhibitor were determined as described earlier.

The stoichiometry of the corn trypsin inhibitor from fraction A was estimated from the reaction of trypsin enzyme with aliquots of fraction A (Fig. 8). From Fig. 8 it can be seen that the extrapolated amount of inhibitor required to give 100 percent inhibition of trypsin was 8.6 ug. In other words, 8.6 ug of inhibitor would be required to completely inhibit 20 ug of trypsin. Using 23,300 (50) as the molecular weight of trypsin and 10,500 as the molecular weight of the inhibitor from fraction A, the calculated stoichiometry is 0.94 (INH.)/1.0 (TRY.). This is lower than, but close to, a one to one ratio of inhibitor to trypsin.

Fig. 8. Plot of the reaction of 20 ug trypsin enzyme with fraction A. Dashed line is extrapolated from the linear portion of the curve.

FIG. 9


7.0
6.0
5.0
4.0
3.0
2.0
1.0
Δ ABS. MIN⁻¹ X10²
2.0
4.0
6.0
8.0
10.0
12.0
ug OF INHIBITOR

Discussion

Trypsin inhibitor was isolated from opaque-2 (1974) corn by the use of trypsin-Sepharose 4B affinity chromatography. The active inhibitor effluent from the affinity column contained 89 percent of the trypsin inhibitor activity in the crude corn extract (Table 1). The active fraction from the trypsin-Sepharose column showed the presence of two major bands and one minor band on analytical disc gel electrophoresis (Fig. 4). Isolation of a homogeneous active inhibitor fraction (Fraction A) was accomplished by DEAE cellulose chromatography, run in the presence of 8 M urea.

Some of fraction A was re-bound to the trypsin affinity column. However, the active fraction collected after being re-bound to the affinity column showed two major bands on analytical disc gel electrophoresis, identical to the two major bands seen in the original trypsin-Sepharose 4B gels (Fig. 4). These data suggest that the two main bands observed on gel electrophoresis of the active fraction from the trypsin-Sepharose column do not represent two different trypsin inhibitors from corn seeds, but rather one inhibitor which has been modified chemically by the trypsin affinity column. SDS gels (Fig. 6) showed the upper band, or band 1 (Fig. 4), to be one single polypeptide, and the lower band, or band 2, to be made up of two polypeptide chains.

The data from the re-chromatography on the trypsin-Sepharose affinity column and the SDS gels indicate that the trypsin-Sepharose 4B column may be modifying the inhibitor from corn seeds by a mechanism similar to the one proposed by Laskowski (33). Laskowski showed that the interaction of "virgin" soybean inhibitor with the enzyme trypsin results

in the production of a modified inhibitor that has one peptide bond cleaved but remains intact due to disulfide bonds (see Diagram 1).

The PMB experiment indicates that the inhibitor's sulfhydryl amino acids are probably disulfide bonded. If the inhibitor was modified by the trypsin as proposed by Laskowski (33), then disulfide bonding would explain why band 2 (Fig. 4) on nonSDS gels appears as a single band but when reduced with B-mercaptoethanol and run on SDS gels appears as two bands.

The chemical similarities of bands 1 and 2 (Fig. 4) is further demonstrated by the comparability of the amino acid composition of fraction A to the amino acid composition of fraction B (Table 3).

Hochstrasser (7) also reported that his corn inhibitor was modified by his trypsin resin by the cleavage of the Arg-Leu bond (Diagram 2) in the inhibitor molecule. However, when he washed the trypsin resin with 6 M urea, only the modified form of the inhibitor was isolated.

In comparing the trypsin inhibitor from opaque-2 (1974) corn (fraction A) to Hochstrasser's (7,22) corn trypsin inhibitor, one notices both similarities and differences. Hochstrasser reports a molecular weight of 19,500 by Sephadex G-75 and ultracentrifuge studies and a molecular weight of 6,981 from his amino acid sequence analyses (7,22). Opaque-2 trypsin inhibitor has a molecular weight of about 10,000. The amino acid composition of Hochstrasser's inhibitor (7) is very similar to that of opaque-2 corn trypsin inhibitor (Table 3). However, Hochstrasser does not report any tryptophan in his corn trypsin inhibitor, while the opaque-2 trypsin inhibitor definitely contains tryptophan, as shown by the absorption spectrum in Fig. 8. The amino acid composition based on Hochstrasser's sequence (22) shows striking

differences when compared to the amino acid composition of the opaque-2 trypsin inhibitor, and even to his own reported amino acid composition (Table 2,7).

In comparing Hochstrasser's amino acid composition (7, Table 2) to the amino acid composition of the opaque-2 trypsin inhibitor, it appears that they may be the same inhibitor molecule. The difference in molecular weight and sequence data will only be resolved with a good molecular weight determination and sequencing of the opaque-2 trypsin inhibitor.

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ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF A TRYPSIN INHIBITOR FROM CORN (*ZEA MAYS* L.) SEEDS

by

Michel J. Swartz
B.S. Boise State College, 1973

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

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Biochemistry

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1975

Proteinase inhibitors are widely distributed in the plant kingdom. Seeds from the Leguminosae and Graminae families and tubers from the Solanaceae family are excellent sources of proteinase inhibitors. The function of proteinase inhibitors in plants is not clearly understood, and little is known about the processes that control the existence of proteinase inhibitors within plants.

Halim and Mertz have shown that corn kernels containing the opaque-2 gene contain more trypsin inhibitor than corn kernels containing the floury-2 gene or normal gene. In this study trypsin inhibitor was isolated from opaque-2 (1974) corn seeds by salt extraction and chromatography on a trypsin-Sepharose affinity column. Analytical disc gel electrophoresis showed the active fraction from the trypsin-Sepharose column to be heterogeneous. The slowest moving component, as seen on the analytical disc gels, was resolved by DEAE chromatography in the presence of 8 M urea. Re-chromatography of this component on the trypsin affinity column resulted in the production of an active trypsin inhibitor fraction that again was heterogeneous, as shown by analytical disc gel electrophoresis. SDS gel electrophoresis indicated the slowest moving component of the analytical disc gel to be a single peptide. SDS gels also indicated that the second slowest moving component on analytical disc gels was composed of two polypeptide chains. This evidence suggests that the affinity column chemically modified the corn inhibitor by a mechanism similar to the one proposed by Laskowski.

Characterization of the corn trypsin inhibitor fraction from DEAE chromatography gave the following results: The corn trypsin inhibitor has a molecular weight of about 10,500. The inhibitor molecule probably has a small amount

of carbohydrate attached to it, as indicated by the phenol-sulfuric acid test. Amino acid analysis showed the corn trypsin inhibitor molecule to contain a high content of proline and half-cystine, and a small amount of tryptophan. p-Mercuribenzoate did not react with the trypsin inhibitor molecule, indicating that there are no free sulfhydryl groups in the corn inhibitor. The stoichiometry of the reaction of corn trypsin inhibitor with trypsin was calculated to be 0.94/1, which is close to a one to one ratio of inhibitor to trypsin.

Comparison of the opaque-2 (1974) corn trypsin inhibitor to the corn trypsin inhibitor of Hochstrasser reveals similarities and differences. A large discrepancy occurs between the reported SDS molecular weight of opaque-2 corn inhibitor and the reported molecular weights of Hochstrasser's inhibitor. The amino acid composition for opaque-2 inhibitor appears to be very similar to the amino acid composition of Hochstrasser's inhibitor. However, marked differences can be noted between the amino acid composition of the opaque-2 corn inhibitor and the amino acid composition based on Hochstrasser's sequenced corn inhibitor. Opaque-2 corn inhibitor contains tryptophan, while Hochstrasser does not report tryptophan in his corn inhibitor.