TRYPSIN INHIBITORS OF SPINACH AND ALFALFA LEAVES

by

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INTRODUCTION

Since the isolation of a trypsin inhibitor from soybean seeds by Kunitz in 1945 (28), similar inhibitors have been found in many other plants (49). There has been much speculation concerning the physiological significance of the plant proteinase inhibitors, both to the plant and to the animal consuming them. Leaf crops, such as alfalfa, are a possible source of considerable amounts of protein for direct animal and human consumption (13,41). It has been reported that alfalfa meal has a growth depressing effect on chicks (12). The trypsin inhibitor content might be responsible for a part of this effect, since isolated soybean trypsin inhibitor depresses rat growth (43). Spinach is a popular green-leaf vegetable for human consumption. It also contains a trypsin inhibitor (7).

This report describes the isolation and purification of trypsin inhibitors from spinach and alfalfa, and presents their differences. Another objective of the study was to determine changes in inhibitor content during the growth of alfalfa.

REVIEW OF LITERATURE

Trypsin inhibitors are proteinaceous substances which occur widely in nature, and which inhibit the proteolytic activity of trypsin (28). In the animal kingdom, trypsin inhibitors are found in ascaris, egg white, bovine colostrum, milk, mammal blood serum, mammal pancreas, urine, etc. In the plant kingdom, they are

distributed in many legume seeds and leaves, potato tuber and leaves, alfalfa, cereal grain and beets (32). Legume seeds, Irish potato, and sweet potato are high in trypsin inhibitor. Fruits generally are low in activity, and leaf tissues such as spinach, broccoli and Brussels sprouts are intermediate (7).

The molecular weights of trypsin inhibitors usually range from 5,000 to 60,000, with most being less than 20,000 (6). With the exception of certain inhibitors from serum, all apparently contain cystine and proline. Many do not contain tryptophan. Optical rotatory dispersion and circular dichroism studies of various inhibitors show that some of them contain helical structure.

The reactive site of trypsin inhibitors consists of a trypsin-accessible arg-X or lys-X bond contained within a disulfide loop (30). It has been reported that Kunitz soybean, Kazal bovine, ovine pancreas, chicken ovomucoid, wheat, rye, corn and peanut inhibitors are arg-X bond while Kazal porcine, canine, human pancreas, Kunitz bovine pancreas, bovine colostrum, potato IIa and IIb, Bowman-Birk soybean, and lima bean are lys-X bond (9). The arg-X inhibitors are less effective inhibitors than lys-X inhibitors (29). In addition, most trypsin inhibitors have a single reactive site, but some of them have double headed reactive sites, such as Bowman-Birk soybean, lima bean, garden bean, and black-eyed pea inhibitors (27).

The disulfide linkage may contribute to the rigidity of the

structure and consequently to the extremely high stability of these low molecular weight inhibitors (52,39). The inhibitors with low molecular weight and high disulfide content are stable toward extremes of pH, temperature and some denaturants (3,16). Cystine accounts for as much as 20 percent of the total amino acid composition of inhibitors (9). Full reduction of disulfide bonds in the inhibitor induces complete loss of inhibitory activity and causes conformational changes. The latter probably is the rate-limiting step (52).

Soybean contains at least four different inhibitors (42), lima bean contains six isoinhibitors (20) and chick pea has six isoinhibitors (39). The presence of multiple proteinase inhibitors having similar or different structure or functions in the same plant or animal tissue has been ascribed to genetic heterogeneity (23,51) or to differences in amide content (15).

The growth-depressing effect of unheated soybean (4), lima bean (48), navy bean (24), alfalfa (18,44) and other legumes (31) on rats or chicks is well known and may be due partially to the trypsin inhibitor present in raw material. Besides the inhibition of growth, chickens exhibit decreased egg production (19,31), and rats show an enlargement of the pancreas and excess enzyme secretion. Some investigations have indicated that inhibitors block protein hydrolysis, reduce the protein efficiency ratio, and stimulate pancreatic enzyme secretion, thus causing the growth depression and pancreatic

hypertrophy (18,35,43). The inhibitors also increase the metabolic conversion of methionine to cystine and the utilization of cystine for protein synthesis (1).

Recently Ryan found that wound-induced accumulation of trypsin inhibitors in potato and tomato leaves is mediated by a wound hormone called proteinase inhibitor-inducing factor (PIIF). The latter is released from wounded tissues and travels through the vascular system to both nearby and distal tissue (17,45). This response is thought to be a defense mechanism directed toward the intestinal proteolytic digestive enzymes of invading insects or toward extracellular proteinases of invading microorganism (46). A survey confirms that substances with PIIF-like activity are widespread in the plant kingdom (50).

Although trypsin inhibitors of plant origin have been studied intensively in recent years, little is known about those present in green plant tissue such as spinach and alfalfa. Borchers et al. found a trypsin inhibitor in alfalfa seeds (5). Kendall found a trypsin inhibitor in aqueous extracts of fresh alfalfa (26). Subsequently, Mitchell et al. isolated a trypsin inhibitor from commercial dehydrated alfalfa which was a polypeptide-carbohydrate complex, was non-competitive, and was slowly heat labile (2,8,44). Mooijman isolated an inhibitor from alfalfa leaves which was a saponin-peptide or a saponin-amino acid complex was stable between pH 2 and 12, and was extremely thermostable (38). Obviously, these two are

different inhibitors. The physiological functions of trypsin inhibitors of green tissues are still obscure, and more studies are needed to determine their role in the plant.

EXPERIMENTAL

Trypsin Inhibitor Assay

The inhibitory activity of the inhibitor was determined by the method of Erlanger (14). In this method, N-benzoyl-DL-arginine-p-nitroanilide (BAPA) is used as a substrate for trypsin. When BAPA is hydrolyzed by trypsin, one of the products is p-nitroaniline, having a yellow color. The activity of trypsin is estimated by measuring the rate of change in absorbance at 410 nm, and inhibitory activity by depression of the change when the inhibitor is added.

Enzyme Solution: 25 mg trypsin were dissolved in 50 ml 0.001 M HCl.

Buffer Solution: 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$. 6.075 g Tris (trishydroxymethylaminomethane) and 2.2 g $CaCl_2$ were dissolved in distilled water, adjusted to pH 8.2 with HCl, and diluted to 1 l.

Substrate Stock Solution: 43.5 mg DL-BAPA were dissolved completely in 1 ml dimethylsulfoxide, and the solution was brought to 100 ml with buffer solution.

Assay System: Two assays were necessary to determine trypsin inhi-

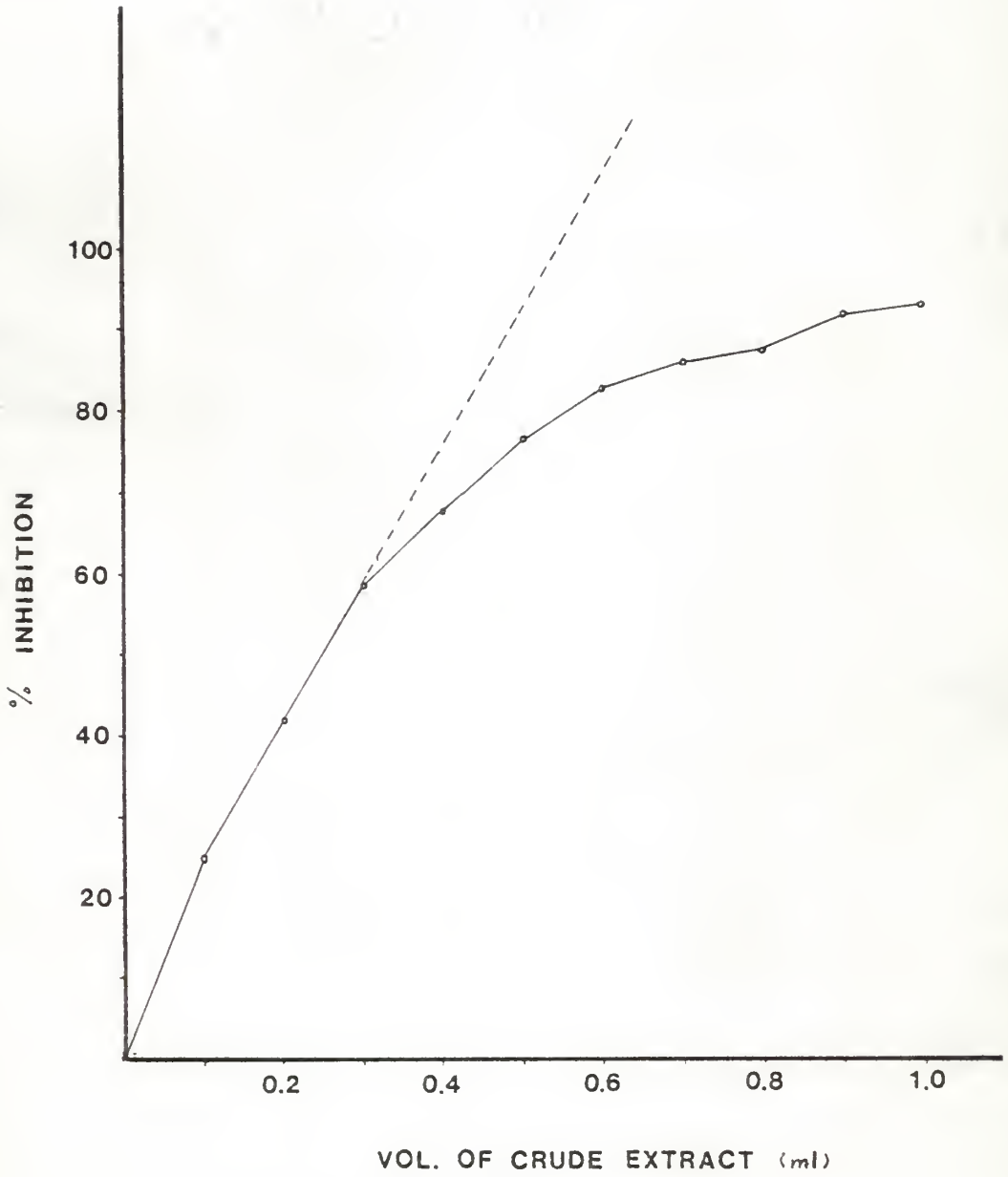
bition. In the reference assay, 1 ml of trypsin and 1 ml of water were added to 2 ml of buffer solution, and the mixture was allowed to incubate in a thermostatically controlled bath at 25°C for 10 minutes. At zero time, 5 ml of substrate stock solution were added and the reaction was allowed to run for 10 minutes. A suitable control without enzyme was also prepared to serve as a blank. Addition of 1 ml of 30% acetic acid terminated the reaction, and the quantity of p-nitroanilide was estimated at 410 nm with a Beckman Spectrophotometer. To measure the inhibitor activity, 1 ml of inhibitor solution was substituted for the 1 ml of water, and enzyme activity was measured as above.

Inhibitory activity was estimated from the residual trypsin activity in the reaction mixture containing the inhibitor, and was expressed as percent inhibition (I) by using the equation

$I\ (\%) = \frac{T-T'}{T} \times 100$, where T' and T are the respective activities of trypsin with and without the inhibitor.

Fig. 1 shows the inhibition pattern resulting from increasing amounts of spinach inhibitor. It can be seen that there is an approximate linear relationship below 50 percent inhibition. Therefore, one trypsin inhibition unit was defined as the amount of inhibitor which causes 50 percent inhibition of trypsin activity under the above assay conditions.

Fig. 1. Inhibition of trypsin activity as a function of the level of crude spinach extract, using BAPA as substract. Dashed line is the extrapolation of the linear portion of the curve.


100
80
60
40
20
% INHIBITION
0.2
0.4
0.6
0.8
1.0
VOL. OF CRUDE EXTRACT (ml)

Protein Determination

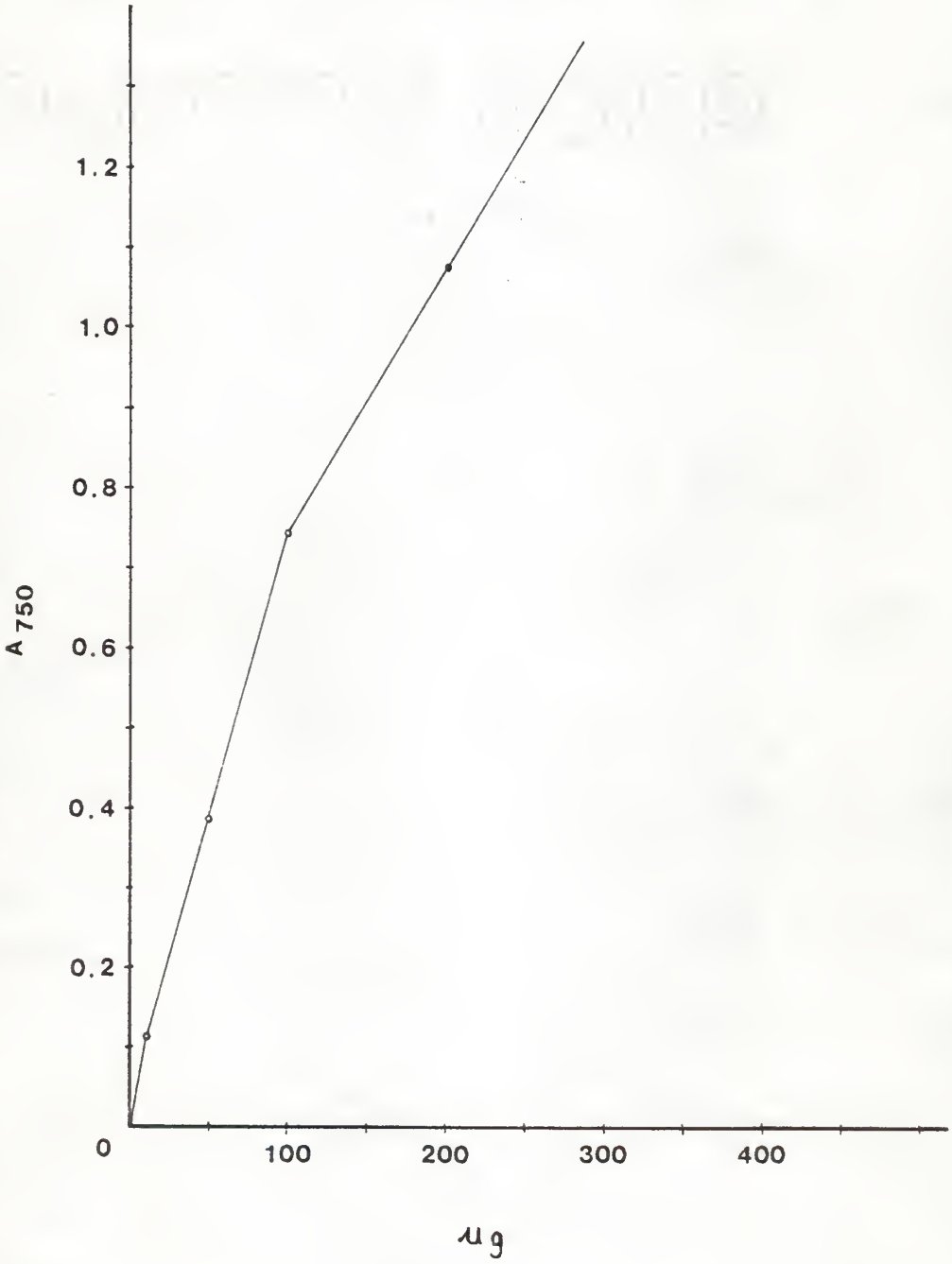
The protein contents of inhibitor preparations were determined by the Lowry method (34). Reagent A was 2 percent Na_2CO_3 in 0.1 M NaOH. Reagent B was 0.5 percent $CuSO_4 \cdot 5\ H_2O$ in 1 percent sodium or potassium tartarate. Reagent C was prepared by mixing 50 ml of Reagent A with 1 ml of Reagent B. Reagent D was commercial Folin-Ciocalteu reagent diluted 2-fold with distilled water. The procedure was as follows: Add 1 ml of Reagent C to 0.2 ml of the protein solution containing 5 to 100 ug of protein. Mix well and let stand 10 minutes or longer at room temperature. Add 0.1 ml of Reagent D very rapidly and mix within a second. Let stand 30 minutes or longer, and measure absorbance with a Beckman Spectrophotometer at 750 nm. Using this procedure, a solution of bovine serum albumin was used to prepare the standard curve shown in Fig. 2.

Isolation and Purification of Trypsin Inhibitor of Spinach

Extraction of Inhibitor

Fresh spinach, purchased at a supermarket, was dehydrated and defatted by two extractions with acetone. The dried spinach was ground with a burr mill, the meal was extracted twice with acetone, and the residue was air dried. The extractions and all subsequent operations were performed at 4 °C.

Fig. 2. Standard curve for spectrophotometric determination of protein. Bovine serum albumin was used as a standard protein.


1.2
1.0
0.8
0.6
0.4
0.2
0
A 750
100
200
300
400
µg

The spinach meal (200 g) was stirred for 4 hours with 1 l of 0.2 M NaCl. The extract was removed by filtering under vacuum. The residue was re-extracted with 600 ml of 0.2 M NaCl, with stirring for 4 hours. The two extracts were combined and centrifuged at 10,000 x g for 15 minutes with a Beckman Model J-21 refrigerated centrifuge. The supernatant was adjusted to 0.05 M Tris-HCl, pH 8.2, 0.02 M $CaCl_2$ and 1 M NaCl. This solution was centrifuged at 10,000 x g for 30 minutes. The supernatant then was subjected to affinity chromatography.

Affinity Chromatography

Affinity chromatography was introduced as a method of enzyme purification by Cuatrecasas et al (10). It is a method for the direct isolation of naturally occurring proteinase inhibitors on the basis of specific complex formation between enzyme and inhibitor. The inhibitor to be purified is passed through a column containing a cross-linked polymer or gel to which the appropriate enzyme has been covalently attached. Elution of the bound inhibitor is readily achieved by changing such parameters as salt concentration or pH, or by addition of a competitive enzyme in solution. Trypsin affinity columns have been used previously for isolating other trypsin inhibitors (33,40).

Fifteen g of cyanogen bromide-activated Sepharose 4B was suspended in 300 ml of 0.001 M HCl for 15 minutes. The gel was washed

with 500 ml of 0.001 M HCl in a sintered glass funnel. The washed Sepharose was transferred to an Erlenmeyer flask with 0.02 M borate buffer, pH 9.0, containing 0.02 M $CaCl_2$. One g of trypsin was dissolved in 5 ml of 0.001 M HCl and added to the Sepharose solution. The mixture was shaken gently at 4 °C for 24 hours by means of a horizontal rotating shaker. Then the suspension was filtered on a sintered glass filter and the gel was washed thoroughly with 0.001 M HCl to remove excess trypsin. The trypsin-Sepharose 4B gel then was transferred to a column (6.5 x 5 cm), and equilibrated with buffer (0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$ and 1 M NaCl). The high concentration of sodium chloride was used to minimize nonspecific or electrostatic binding of non-inhibitor molecules in the spinach crude extract.

The clarified crude inhibitor preparation was applied to the column at a flow rate of 140 ml per hour, and 10 ml fractions of the effluent were collected. After all the crude extract had been applied to the column, the starting buffer again was applied to the column. The column was washed thoroughly until the effluent absorbance at 280 nm had decreased to less than 0.025. Finally, the inhibitors were displaced by passing an eluting solvent (0.1 M glycin-HCl buffer, pH 1.5) through the column. The eluting buffer was collected with a fraction collector, and the protein content of each fraction was estimated by A_{280} measurements.

Upon chromatography of the crude inhibitor preparation on the

trypsin-Sepharose 4B column, inactive proteins and pigmented material did not bind to the column and were washed from the column by the adsorption buffer (See Fig. 3). There was one peak with inhibitory activity in the effluent after washing with glycine-HCl buffer. The recovery of inhibitory activity at this stage was 73.2 percent of the activity of the crude extract.

Polyacrylamide Disc Gel Electrophoresis

The active fraction from the trypsin-Sepharose 4B column was examined for homogenity by analytical polyacrylamide disc gel electrophoresis according to the procedure of Davis (11). It was performed at room temperature. Gels were stained with 0.5 percent amido black in 7 percent acetic acid and 40 percent ethanol for thirty minutes. They were destained with 7 percent acetic acid and 40 percent ethanol with the aid of Dowex-1 anion exchange resin.

The inhibitor fraction was subjected to 7.5 percent polyacrylamide gel electrophoresis. There were three protein bands on the electrophoresis gel, two major and one minor, as shown in Fig.4. This result indicated that the active fraction from the trypsin-Sepharose 4B column was heterogeneous. Thus, other chromatography was run in attempts to resolve the inhibitor.

Sephadex G-10 Chromatography

A 3.5 x 45 cm chromatographic column was used. Dry Sephadex

Fig. 3. Elution profile of spinach trypsin inhibitor on a trypsin-Sepharose column. The adsorption buffer was 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$ and 1 M NaCl. The eluting buffer was 0.1 M gl glycine, pH 1.5.

—o—o— Absorbance

--x--x-- Inhibitory activity

⊢——⊣ Fraction combined for use in subsequent experiment.

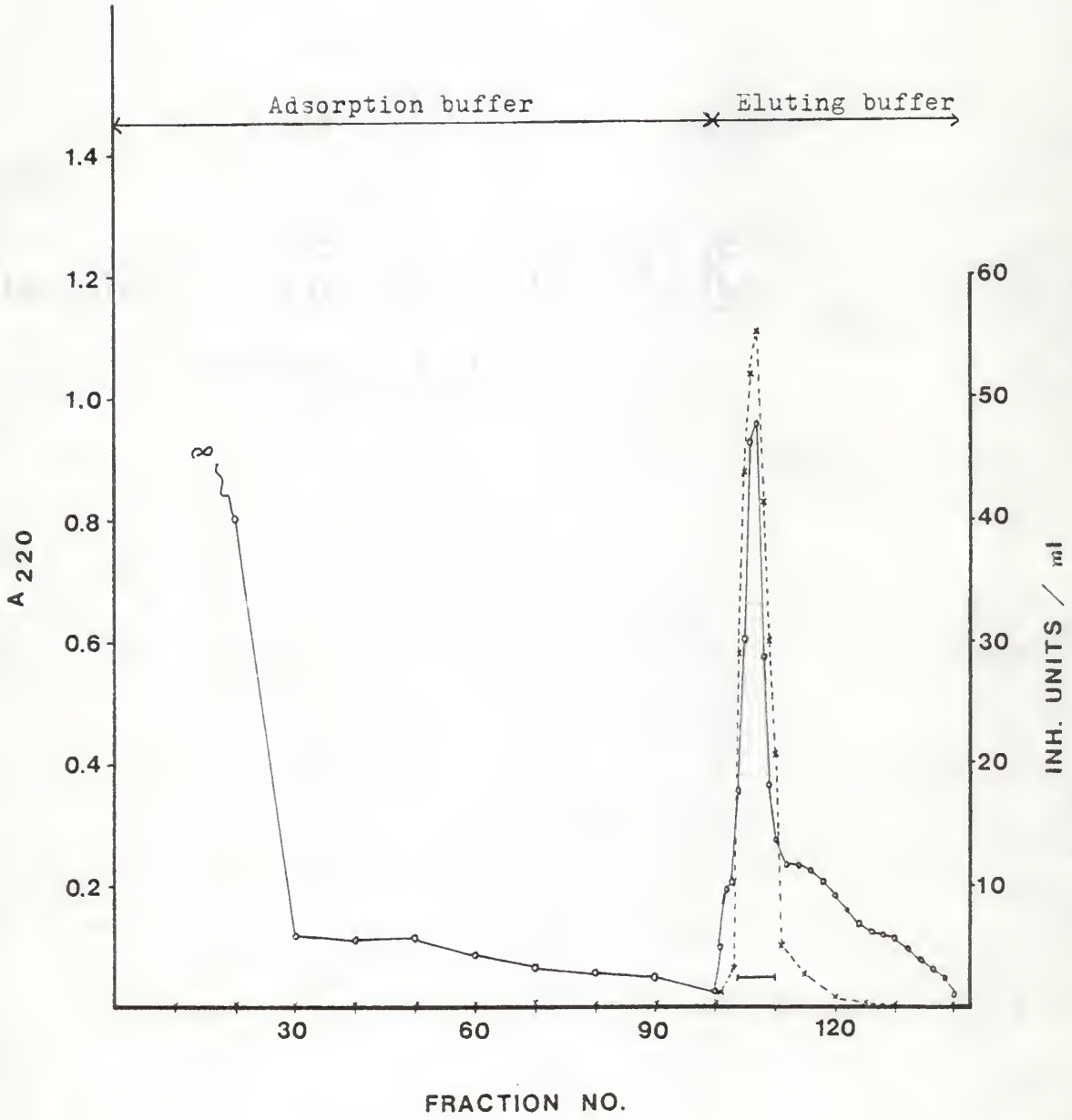

Adsorption buffer
Eluting buffer
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A 220
60
50
40
30
20
10
INH. UNITS / ml
30
60
90
120
FRACTION NO.

Fig. 4. Disc gel electrophoresis pattern of the active fraction from the trypsin-Sephrose 4B column.

G-10 (20 g) was suspended in 500 ml of water on a boiling water bath for 3 hours to eliminate air bubbles in the gel slurry and to accelerate the swelling rate (41). The swelled gel was placed in the column and equilibrated with 0.01 M Tris-HCl buffer, pH 7.5. The inhibitor solution obtained from the trypsin-Sepharose column was applied to the column and elution was accomplished with the same buffer at a flow rate of 70 ml per hour. The eluate was collected in 4.5 ml fractions. Absorbance of each fraction was measured with a Beckman spectrophotometer at 220 nm, and trypsin inhibitory activity was assayed by the procedure described earlier. The elution profile (Fig. 5) had two peaks indicating the presence of two components. The first component to elute contained all the inhibitory activity. The second, and minor component, was inactive, and was discarded.

DEAE-Cellulose Chromatography

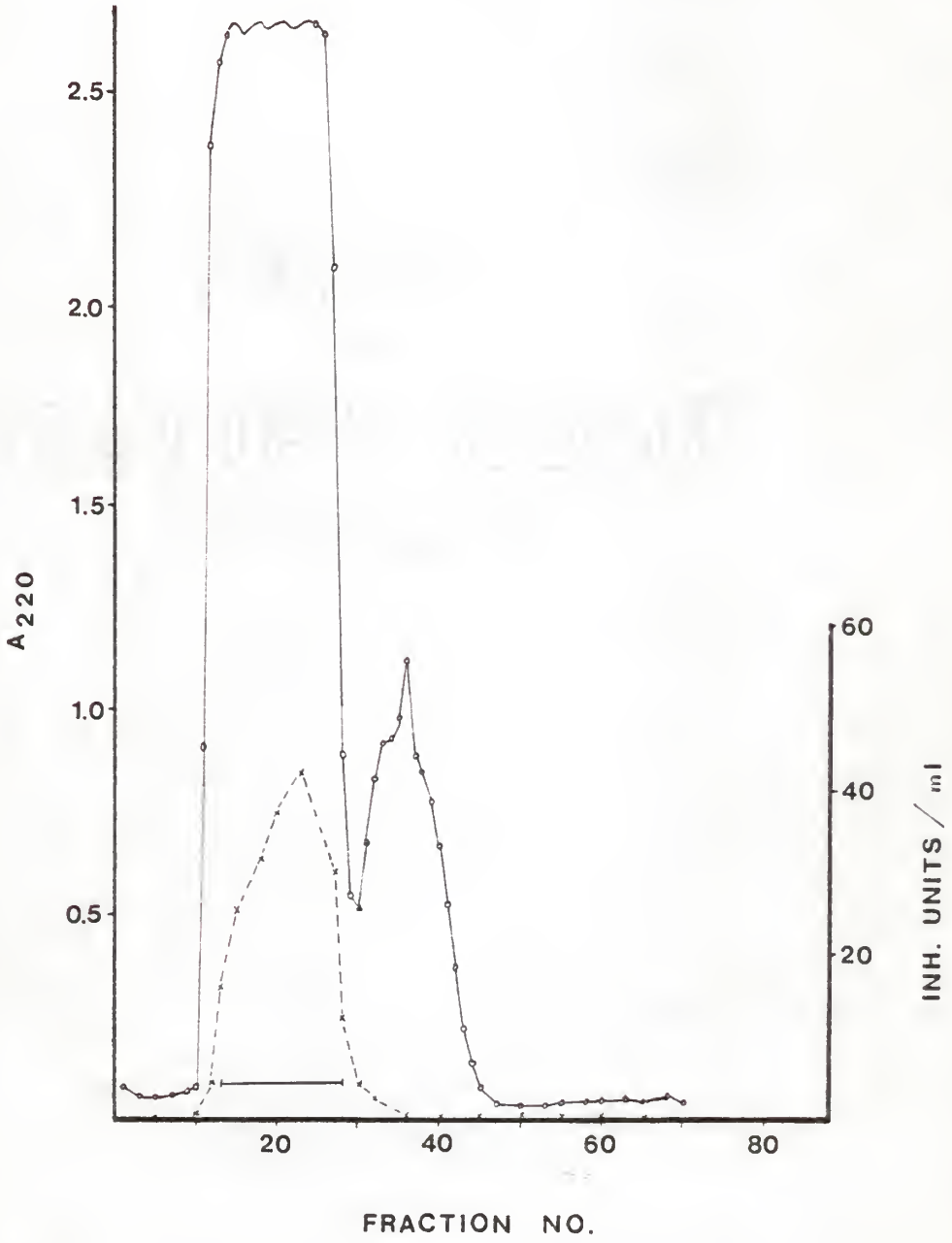
Twenty g of dry DEAE-cellulose were suspended in distilled water and the fine particles were decanted off. Then the gel was soaked in 0.5 M HCl, the suspension was filtered with a sintered glass filter, and the residue was washed with a similar solution until all color was removed. The gel was stirred in 0.5 M NaOH, after which it was filtered and washed free of alkali with distilled water. The DEAE-cellulose gel was equilibrated in 0.01 M Tris-HCl buffer, pH 8.0, and put into a 2.5 x 30 cm column.

Fig. 5. Sephadex G-10 chromatography of the active fraction from the affinity column.

—o—o— Absorbance

--x--x-- Inhibitory activity

⊢———⊣ Fraction combined for use in subsequent experiments.


2.5
2.0
1.5
1.0
0.5
A 220
60
40
20
INH. UNITS / ml
20
40
60
80
FRACTION NO.

The active effluents from the Sephadex G-10 column were combined and applied to this column, and the column was washed with 200 ml of the starting buffer. The inhibitor then was eluted by means of a linear gradient of 0.5 M NaCl in a total volume of 600 ml of 0.01 M Tris-HCl buffer, pH 8.0. The eluate was collected in 2.2 ml fractions. The elution and inhibitor profiles are shown in Fig. 6.

The active component from the Sephadex column separated into two distinct fractions, with some indication that the slower moving fraction was partially resolved into a third fraction. Each component possessed inhibitory activity. Recovery of inhibitory activity from the column was less than half the amount applied. Aliquots of fractions A, B, and C were subjected to 7.5 percent polyacrylamide disc gel electrophoresis, but no bands were observed, perhaps because of the low protein concentration.

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

Fig. 6. DEAE-cellulose chromatography of the pooled material desorbed from Sephadex G-10.

—o—o— Absorbance

--x--x-- Inhibitory activity

⊢———⊣ Fractions combined for use in subsequent experiments.

INH. UNITS / ml
40
30
20
10
NaCl
0.5M
A
B
C
A 220
2.5
2.0
1.5
1.0
0.5
30
60
90
120
150
180
210
FRACTION NO.

Table 1. Purification of Spinach Trypsin Inhibitor

Purification steps	Inhibitor units (ml^{-1})	Volume (ml)	Total Inhibitor units (a)	Protein (mg/ml) (b)	Sp. Act. (mg^{-1}) (c)	Recovery (%) (d)
1. Crude extract	4.1	900	3,690	6.4	0.6	100
2. Trypsin-Sepharose 4B	37	73	2,709	0.98	37.8	73.2
3. Sephadex G-10	29	72	2,146	0.75	38.6	58.2
4. DEAE-cellulose fraction A	16	33	528	0.41	39	14.3
fraction B	8	22	176	0.21	38.1	4.8
fraction C	7.2	15.4	111	0.18	40	3

See the next page for explanation of footnotes.

(a) Total inhibitor units were calculated as:

Inhibitor units x volume

(b) Protein concentration was determined by Lowry method (34).

(c) Specific activity was calculated by:

Inhibitor units/ml divided by protein concentration

(d) Recovery was calculated by:

Total inhibitor units divided by total inhibitor units in the crude extract.

Isolation and Purification of Trypsin Inhibitor of Alfalfa

Fresh alfalfa was obtained from the Dairy Department research farm of Kansas State University. The procedure for preparation of a crude extract was the same as for spinach. Fig. 7 shows that there is a linear relationship between inhibition and inhibitor concentration up to about 75 percent inhibition.

Affinity Chromatography

The crude alfalfa extract was applied to a trypsin-Sepharose 4B column, and the inhibitor was eluted as described earlier. The elution profile (Fig. 8) shows only one peak, which contained all the inhibitory activity. Recovery of the inhibitor applied to the column was 83 percent.

Sephadex G-10 Chromatography

Half of the active portion from the trypsin-Sepharose 4B column was applied to a Sephadex G-10 column. Since the amount of inhibitor was greater than for spinach, half the amount was applied to avoid column overloading. Three peaks were observed in the elution profile, based on A_{220}. However, only one peak had inhibitory activity (See Fig. 9).

CM-Cellulose Chromatography

A chromatographic column of 2.5 x 25 cm was used. Twenty g

Fig. 7. Inhibition of trypsin activity as a function of the level of crude alfalfa extract, using BAPA as substrate. Dashed line is the extrapolation of the linear portion of the curve.

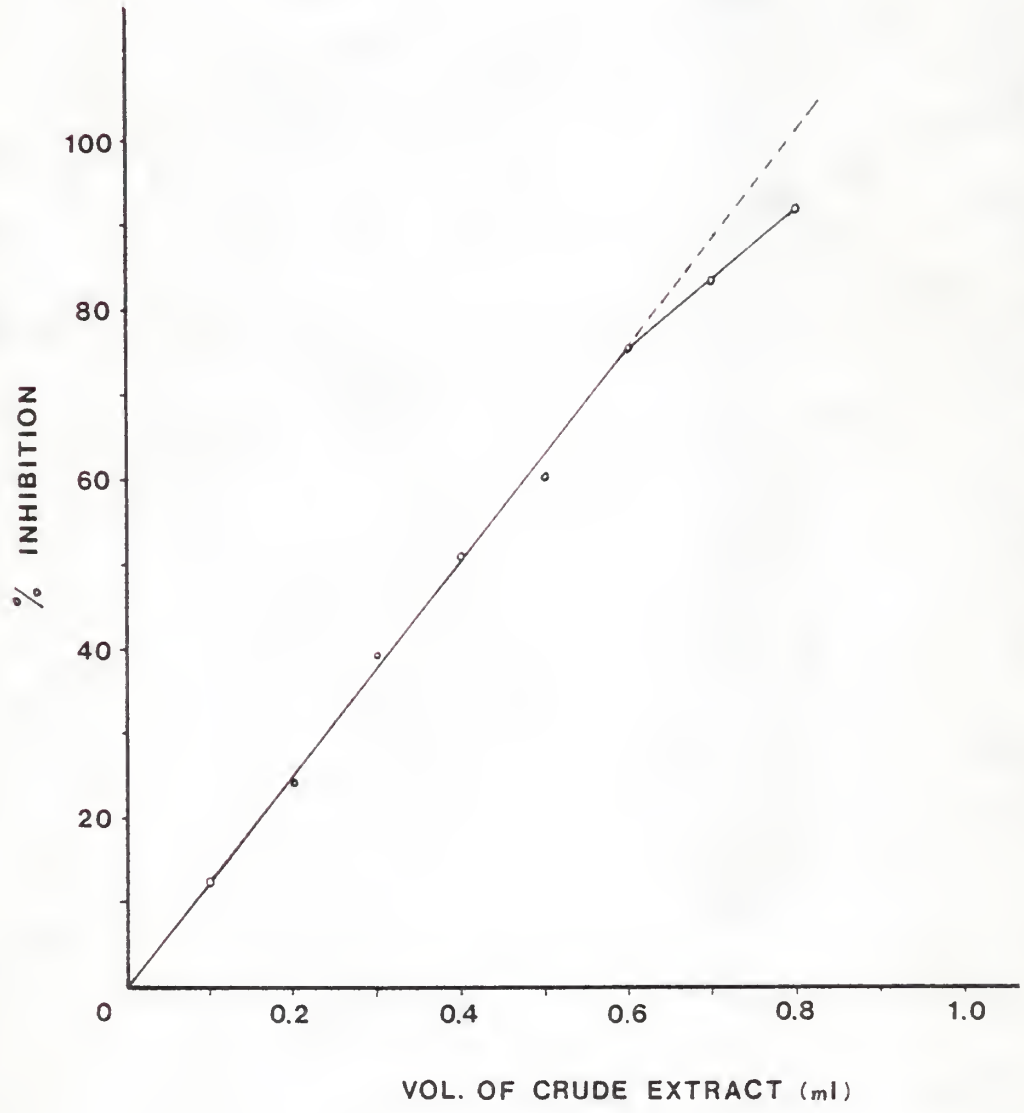

% INHIBITION
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
VOL. OF CRUDE EXTRACT (ml)

Fig. 8. Elution and activity profile of alfalfa trypsin inhibitor on a trypsin-Sepharose column. The adsorption buffer was 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$ and 1 M NaCl. The eluting buffer was 0.1 M glycine-HCl, pH 1.5.

—o—o— Absorbance

--x--x-- Inhibitory activity

⊢———⊣ Fractions combined for use in subsequent experiments.

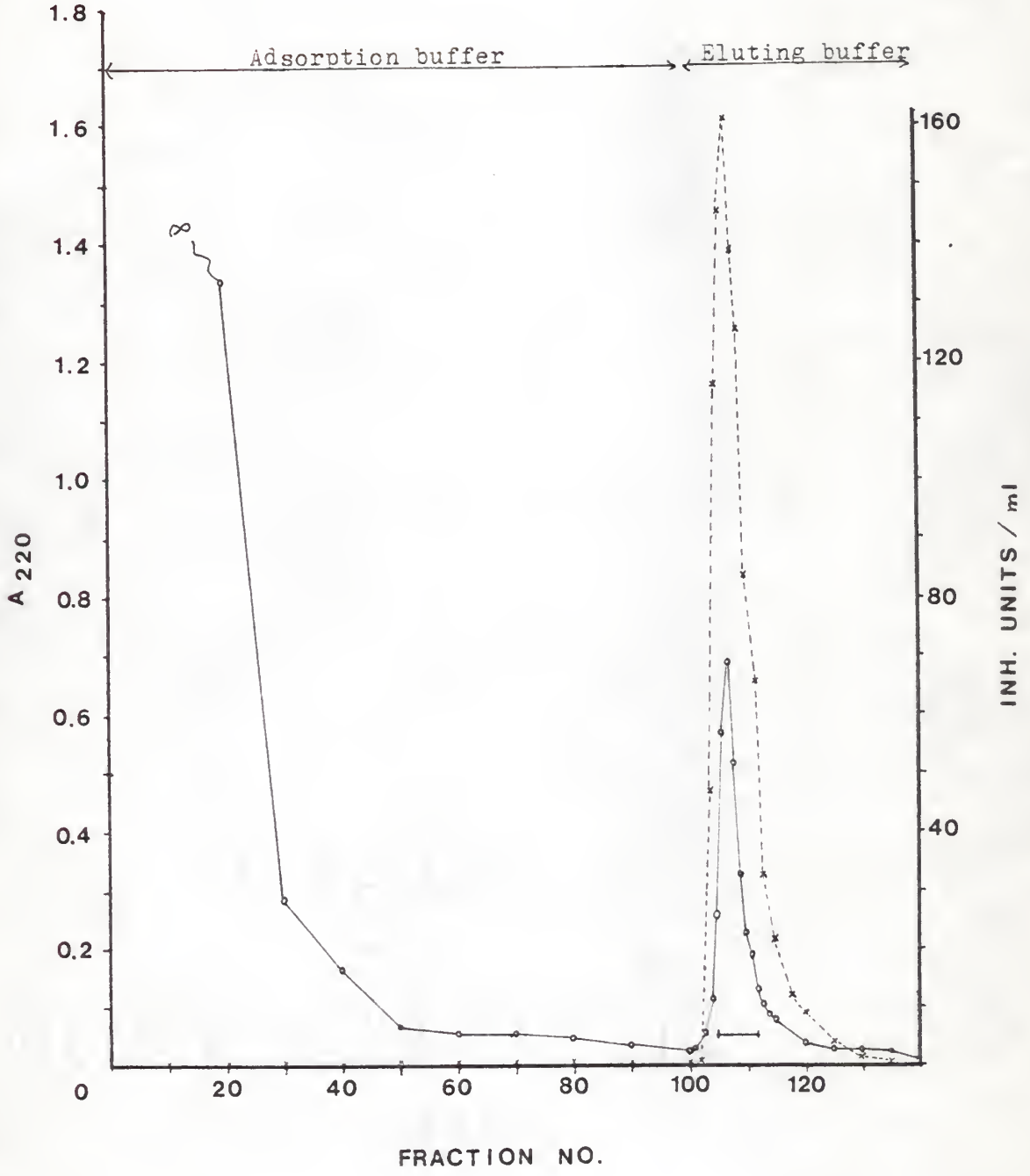
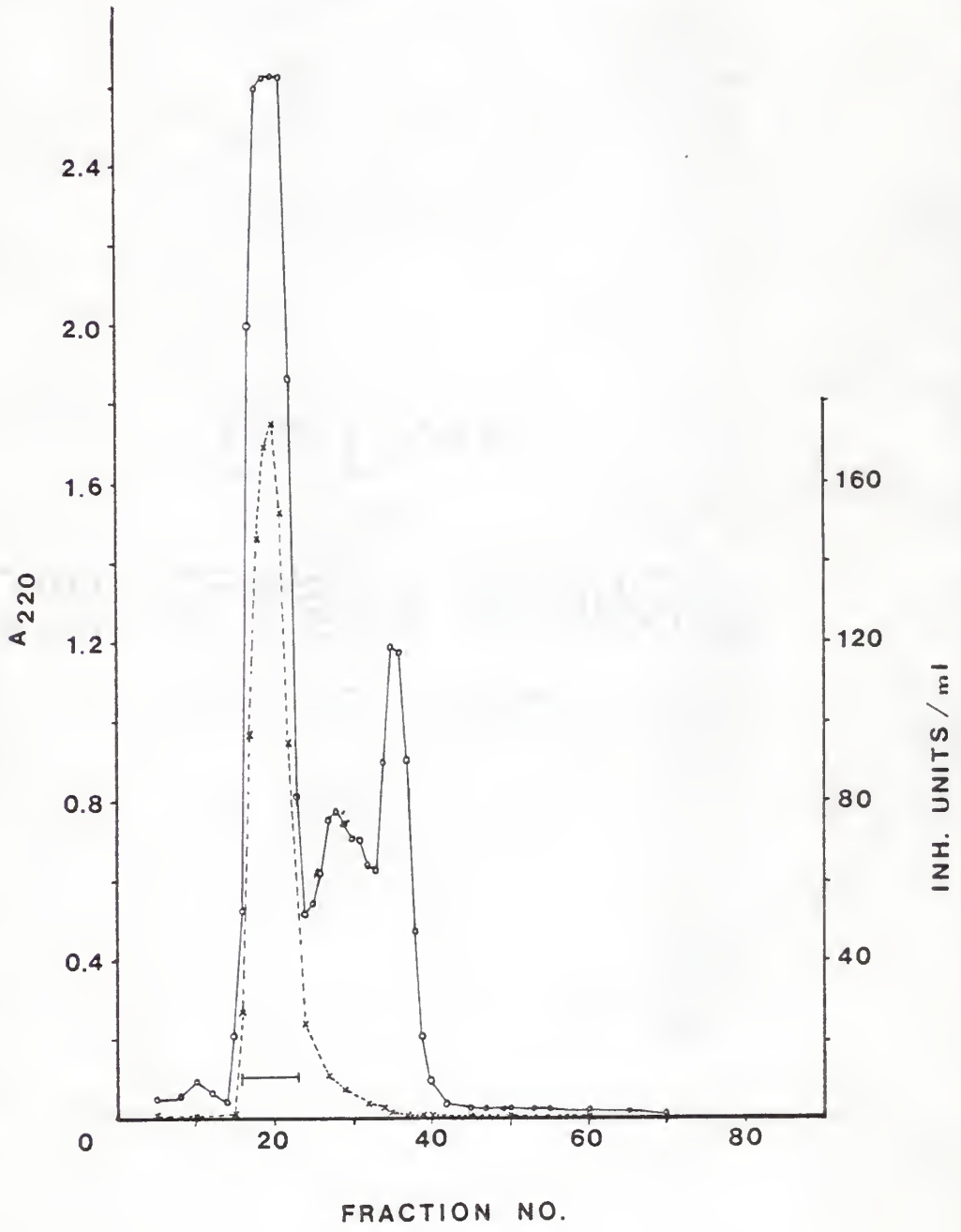

Adsorption buffer
Eluting buffer
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A 220
160
120
80
40
INH. UNITS / ml
20
40
60
80
100
120
FRACTION NO.

Fig. 9. Sephadex G-10 chromatography of the active fraction from the affinity column.

—o—o— Absorbance

--x--x-- Inhibitory activity

⊢———⊣ Fractions combined for use in subsequent experiments.


2.4
2.0
1.6
1.2
0.8
0.4
0
A 220
160
120
80
40
INH. UNITS / ml
20
40
60
80
FRACTION NO.

of dry CM-cellulose was stirred with distilled water, then stirred with 0.5 M NaOH, and then with 0.5 M HCl. The remainder of the procedure was the same as for the preparation of the DEAE-cellulose column. The active fraction from the Sephadex column was chromatographed on the CM-cellulose column. A representative run is shown in Fig. 10. The protein was eluted as a single peak and all the inhibitory activity was present in the fractions within the peak. Recovery was 58.8 percent of the total activity of the crude extra extract.

The progress of purification through the various stages of the process is shown in Table 2. The trypsin-Sepharose column yielded directly a preparation which essentially was pure, as judged by subsequent chromatographic behavior.

Sodium Dodecyl Sulfate Disc Gel Electrophoresis

The active material from the CM-cellulose column was subjected to 15 percent poluacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol (36). The gels were 10 cm long. They were stained with 0.5 percent amido black in 7 percent acetic acid and 40 percent ethanol. Destaining was accomplished with 7 percent acetic acid and 40 percent ethanol. The results are shown in Fig. 11, where one band is seen in the gels from the trypsin-Sepharose 4B and CM-cellulose columns. The location of the band is near the bottom of the gel,

indicating the molecular weight of the alfalfa inhibitor is very low. With this system, only a broad, faint band was obtained, because of a low protein concentration or because the gel system is not suitable for alfalfa inhibitor.

Fig. 10. CM-cellulose chromatography of the pooled inhibitory material desorbed from Sephadex G-10.

—o—o— Absorbance

--x--x-- Inhibitory activity

⊢———⊣ Fractions combined for use in subsequent experiments.

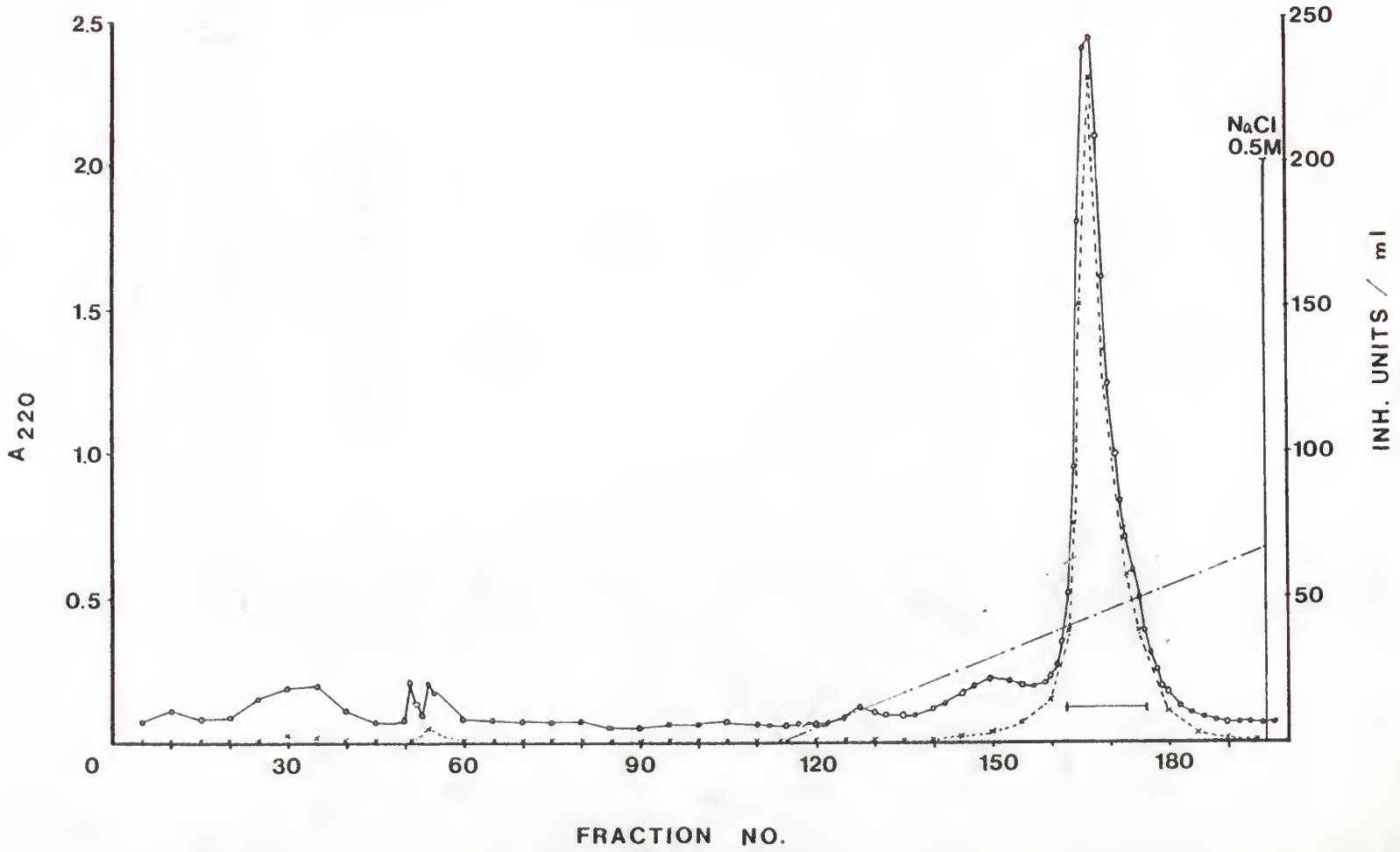

2.5
2.0
1.5
1.0
0.5
0
A 220
30
60
90
120
150
180
FRACTION NO.
250
200
150
100
50
INH. UNITS / ml
NaCl
0.5M

Fig. 11. Electrophoresis in the presence of sodium dodecyl sulfate. Migration was from top to bottom. From left to right: Sepharose 4B active fraction, and CM-cellulose active fraction.

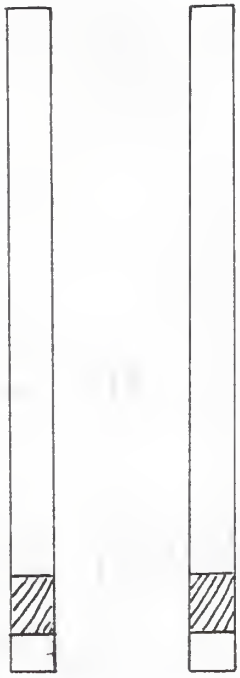


Table 2. Purification of Alfalfa Trypsin Inhibitor

Purification steps	Inhibitor units (ml^{-1})	Volume (ml)	Total Inhibitor units	Protein (mg/ml)	Sp. Act. (mg^{-1})	Recovery (%)
1. Crude extract	7	1,650	11,550	10.5	0.67	100
2. Trypsin-Sepharose 4B	120	80	9,600	1.06	113.2	83.1
3. Sephadex G-10	116	72	8,352	0.95	122.1	72.3
4. CM-cellulose	104	65	6,760	0.85	122.4	58.5

Amino Acid Composition of Alfalfa Trypsin Inhibitor

Analyses were carried out on a Beckman sutomatic amino acid analyzer. The amino acid compositions were derived from duplicate analyses of the inhibitor. The fraction of alfalfa extract after CM-cellulose chromatography was hydrolyzed with 6 N HCl for 20 hours at 110 °C in evacuated, sealed tubes. Half-cystine and methionine were determined as cysteic acid and methionine sulfone after hydrogen peroxide and performic acid oxidation (21). Since the molecular weight of the alfalfa inhibitor was not determined, the amino acid composition presented in Table 3 is the ratios of the amino acid residues. The molecular weight of the alfalfa inhibitor is small, because it can pass through dialysis tubing. The molar percents of the amino acids of alfalfa inhibitors were comparied with the Bowman-Birk soybean trypsin inhibitor based on its sequence data (22).

The amino acid compositions of the two inhibitors differ appreciably. Alfalfa inhibitor contains much more threonine, proline, and isoleucine, and much less lysine, arginine, aspartic acid, glutarmic acid, serine, valine, leucine and tyrosine. Both, however, have high half-cystine contents. Methionine was not detected in alfalfa inhibitor, whereas it is present in soybean inhibitor. Conversely glycine is absent in soybean inhibitor, but is present in alfalfa inhibitor. The higher proline content of alfalfa inhibitor may result in less α-helical structure in this inhibitor.

Table 3. Amino Acid Composition of Alfalfa Trypsin Inhibitor and of Bowman-Birk Soybean Trypsin Inhibitor.

Amino acid	Alfalfa inhibitor		Bowman-Birk STI
	average molar ratio	molar percent	molar percent
Lysine	5.32	4.19	7.81
Histidine	2.48	1.95	1.56
Arginine	2.36	1.86	3.13
Aspartic acid	12.00	9.46	17.19
Threonine	15.10	11.90	3.13
Serine	6.36	5.01	14.06
Glutamic acid	7.37	5.81	10.94
Proline	15.30	12.06	9.38
Glycine	3.03	2.39	0
Alanine	6.09	4.80	6.25
Half-cystine	31.90	25.14	21.88
Valine	1.14	0.90	1.56
Methionine	0	0	1.56
Isoleucine	9.19	7.24	3.13
Leucine	2.18	1.72	3.13
Tyrosine	2.11	1.65	3.13
Phenylalanine	4.98	3.92	3.13

Effect of Stage of Growth on Trypsin Inhibitor Content of Alfalfa

Alfalfa was obtained from the Dairy Department research farm of Kansas State University. Samples were taken when the plants were approximately 5, 10, 15, 20, and 25 inches high. Since a trypsin inhibitor has been reported in alfalfa seeds (5), and since it might be different from the one in leaves and stems, sampling was discontinued when flower buds appeared.

Extraction studies were performed by extracting 10 g of fresh alfalfa with 100 ml of buffer solution (0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) in an Omni-mixer for one minute at full speed. The extract was obtained by centrifugation. The moisture content was measured by vacuum drying at 54 °C overnight. Trypsin inhibitory activities were measured as described earlier.

The results are shown in Fig. 12. It was found that the amount of trypsin inhibitor in leaves and the whole plant decreased initially, and then increased after the second growth stage. Ryan and Shumway reported a similar change in Inhibitor I of potato leaves (47). However, in the case of potato Inhibitor I, the level in the leaf decreased when rhizome development began.

Activity changes in stems was fairly similar to that of leaves, although the initial decrease persisted to the third sampling date, and the subsequent increase was more erratic. However, stems at

a given sampling date had much less inhibitor than leaves at the same sampling date.

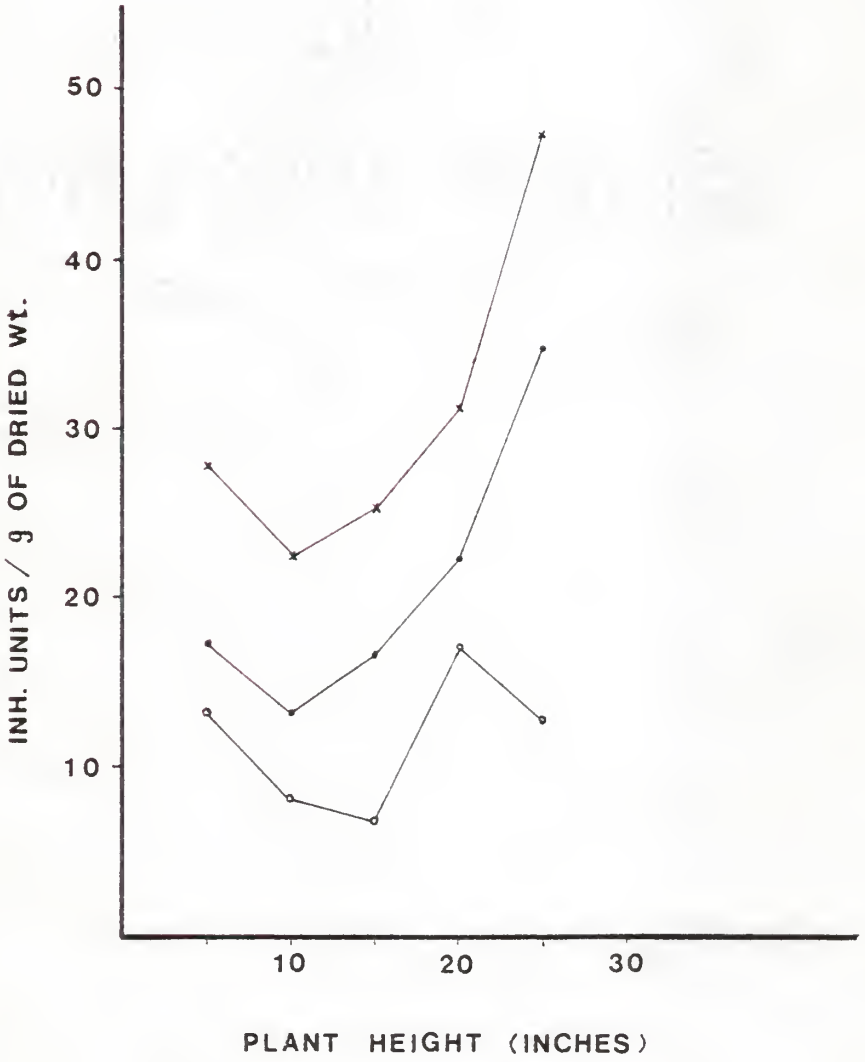
some insect damage to leaves was obvious when the fifth sample was collected. McFarland and Ryan (39,48) found alfalfa to contain proteinase inhibitor-inducing factor activity (PIIF), which caused formation of protease inhibitor when the plant was subjected to insect attack. They suggested that PIIF may be a primitive immune response of the plant toward the intestinal proteolytic digestive enzyme of invading insects. Thus, insect damage to our samples might have contributed to the higher activity found in alfalfa with increasing maturity.

Fig. 12. Trypsin inhibitor activity of alfalfa at five growth stages.

—x—x— Leaves

—•—•— Whole plants

—o—o— Stems


50
40
30
20
10
INH. UNITS / g OF DRIED Wt.
10
20
30
PLANT HEIGHT (INCHES)

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TRYPSIN INHIBITORS OF SPINACH AND ALFALFA LEAVES

by

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AN ABSTRACT OF A MASTER'S THESIS

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A study was made of trypsin inhibitors in the vegetative portions of spinach and alfalfa. The dried and ground tissue was extracted with acetone to remove lipids and chlorophyll. The inhibitors then were isolated by extracting with salt solution and passing the extract through a trypsin-Sepharose 4B affinity chromatography column. They were purified further by Sephadex G-10 chromatography and ion-exchange chromatography.

The inhibitor of spinach was separated into three isoinhibitor by a DEAE-cellulose column. They accounted for about 22 percent of the inhibitory activity of the crude extract.

The inhibitor of alfalfa showed only one protein peak when passed through a CM-cellulose column. The activity of this component accounted for about 59 percent of the activity of the crude extract. The amino acid composition of this fraction indicated that half-cystine is the dominant amino acid residue and accounted for about 25 percent of the amino acid content. It also has higher proline and threonine contents, and no detectable methionine.

A study of inhibitory activity of alfalfa at increasing stages of maturity showed that the amount of trypsin inhibitor in leaves and stems decreased initially, and then increased after the second or third growth stage. Stems at a given sampling date had much less inhibitory activity than leaves. The increased inhibitory activity found in the more mature plants may have resulted from insect damage

which had occurred. McFarland and Ryan found alfalfa to contain a proteinase inhibitor-inducing factor, which caused formation of proteinase inhibitor when the plant was subjected to insect attack.