



The isolation and characterization of a trypsin inhibitor from *Medicago sativa* (alfalfa)
by Richard George Myott

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:

A naturally occurring protein proteinase inhibitor specific for trypsin was shown to be present in the seeds of alfalfa. The isolated inhibitor was characterized in terms of purity, amino acid composition, molecular weight, binding stoichiometry, equilibrium dissociation constant, rate constants of association and dissociation, resistance to denaturing conditions and proteolytic hydrolysis, and the presence of carbohydrate.

Ground defatted seeds were extracted with an aqueous acidic solvent (pH 4.0) at 22°C for 10 hours. The inhibitor (0.24 mg/gm seeds) was removed from the extract supernatant by affinity chromatography using insoluble sepharose-trypsin. Further purification was attempted using gel filtration and ion exchange chromatography on CM cellulose. Lyophilization yielded a white fluffy material.

The inhibitory activity was due to two or more proteins of similar size and charge. Amino acid analysis showed that the purified inhibitor system was comprised of, approximately 58 amino acids. It contained large amounts of half cystine (14) and proline (5), no methionine or tryptophan and only trace amounts of valine. The molecular weight was determined to be 7800 daltons.

The alfalfa inhibitors form complexes with trypsin with a molar binding stoichiometry of 1:1. Complex formation was competitive with an equilibrium dissociation constant of $1.8 \times 10^{-8} M$.

The alfalfa inhibitors are stable to denaturing conditions, including extremes in pH and temperature. They are also stable to hydrolysis by enzymes such as pepsin, chymotrypsin, carboxypeptidase A and carboxypeptidase B.

The inhibitory activity of the alfalfa inhibitor is dependent on disulfide integrity.

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ABSTRACT

A naturally occurring protein proteinase inhibitor specific for trypsin was shown to be present in the seeds of alfalfa. The isolated inhibitor was characterized in terms of purity, amino acid composition, molecular weight, binding stoichiometry, equilibrium dissociation constant, rate constants of association and dissociation, resistance to denaturing conditions and proteolytic hydrolysis, and the presence of carbohydrate.

Ground defatted seeds were extracted with an aqueous acidic solvent (pH 4.0) at 22°C for 10 hours. The inhibitor (0.24 mg/gm seeds) was removed from the extract supernatant by affinity chromatography using insoluble sepharose-trypsin. Further purification was attempted using gel filtration and ion exchange chromatography on CM cellulose. Lyophilization yielded a white fluffy material.

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The alfalfa inhibitors are stable to denaturing conditions, including extremes in pH and temperature. They are also stable to hydrolysis by enzymes such as pepsin, chymotrypsin, carboxypeptidase A and carboxypeptidase B.

The inhibitory activity of the alfalfa inhibitor is dependent on disulfide integrity.

INTRODUCTION

The term "protein proteinase inhibitor" refers to a class of proteins which associate reversibly with one or more proteolytic enzymes to form complexes of discrete stoichiometry in which all of the catalytic functions of the proteinase are competitively inhibited. Plant proteinase inhibitors fall into two general molecular weight classes, one being near 10,000 and the other around 20,000. The plant inhibitors generally contain amino acids only, whereas many inhibitors from animal sources contain varying amounts of carbohydrates, i.e., the ovomucoids and human serum inhibitors. Typically the proteinase inhibitors have high proline and disulfide content, suggestive of their compact structures, and low amounts or total absence of tryptophane, histidine, cystine, and methionine. They are readily soluble and possess unusual stability against denaturing conditions or proteolytic hydrolysis. The inhibited proteinases are usually endopeptidases, i.e., serine proteinases, although in 1968, Rancour and Ryan isolated an inhibitor which inhibited carboxypeptidase B (1).

Proteins that inhibit proteolytic enzymes are often found in high concentration in many seeds and other storage

organs (2). Inhibitor proteins are also found in virtually all animal tissues and fluids (3,6). The plant inhibitors have been the object of considerable research for many years because of their capacity to complex with and inhibit proteolytic enzymes from animals and microorganisms (6). Interestingly, they rarely inhibit proteolytic enzymes from plants, a fact which lends uncertainty to their possible role in plant physiology. The inhibitors have become valuable tools for the study of proteolysis in medicine and biology. They are of particular interest because of their therapeutic potentials in controlling proteinases involved in a number of disorders such as pancreatitis (3), shock (3), and emphysema (4). Recently they have been under study as agents in the regulation of mammalian fertilization (5). Biochemists have also profitably studied plant proteinase inhibitors as model systems to explore the mechanism of action and inhibition of proteinases (2,6). The presence of proteinase inhibitors in important plant foods has also made them the object of great nutritional interest (7,8).

The nutritional aspects of the inhibitors have been covered in a number of reviews (7,8,9), as have the

chemical aspects (2,10). Interest in the pharmacological properties of these inhibitors and their possible clinical use has prompted a symposium on this subject (11). Recently (1973) a review concerned with the physiological importance of the proteinase inhibitors to the plant itself has appeared (12). It appears that the trypsin inhibitors are not important in regulation of endogenous proteinases (13,14). Although they may have a storage role (15), it appears that they function mainly to prevent microbial invasion (16) and attack by insects (17) through inhibition of their respective proteolytic enzymes.

Read and Haas (18), in 1938, found that an aqueous extract of soybean flour inhibited the ability of trypsin to liquify gelatin. The fraction of soybean protein responsible for this effect was partially purified by Bowman (19) and subsequently isolated in crystalline form by Kunitz (20). The existence of an inhibitor of trypsin in soybeans, which could be inactivated by heat, seemed to offer a reasonable explanation for the observation that heat treatment improved the nutritive value of the soybean proteins (21). The realization that proteinase inhibitors might be of nutritional significance in plant foodstuffs,

stimulated a search for similar factors in other plant materials. This search showed that the occurrence of proteinase inhibitors in plant storage organs, such as seeds and tubers, is widespread. They have been isolated mainly from the families of Leguminosae, Graminae, and Solanaceae, but also are found in a number of other families, and in fact probably appear ubiquitously throughout the plant kingdom (22).

The inhibitors contain "active sites" for the inhibition of proteolytic enzymes that endow them with their specificity (2,6). The "trypsin-specific" inhibitors always have either a lys-x or arg-x sequence at the binding site (2), whereas chymotrypsin-specific inhibitors usually have a leu-x at their active centers (23). These sites are part of a very large binding area that is necessary for the proper structural alignments that confer unusual stability to the enzyme-inhibitor complex (24).

The mechanism of inhibition has not yet been satisfactorily explained. Laskowski and co-workers (2) have hypothesized a requirement for a serine-acyl intermediate between the enzyme and inhibitor. Alternatively, Ryan (25,25a) and Feeney (26) have shown that inactive chemical

derivatives of the enzymes also bind inhibitors and form complexes that could not involve an acyl-intermediate. Huber et al. (27), using X-ray crystallography, has shown that in the case of bovine pancreatic trypsin inhibitor, the trypsin-inhibitor complex involves formation of a tetrahedral intermediate at the carbonyl carbon of the inhibitor active site residue which persists in the crystalline structure. Both covalent and hydrogen bonding were observed to play a role in stabilizing the tetrahedral geometry of the active site complex. Formation of the tetrahedral intermediate cannot be generally required, however, in view of the studies of Ryan and Feeney (cited above).

The emerging picture from structural and specificity similarities among plant inhibitors from different sources indicates that the active sites of the inhibitors may have been conserved over millions of years of evolution. This suggests that the inhibitory capacity and possibly their presently unrecognized functions occupy an essential role within the physiology of the plant.

Nutritional Significance of Alfalfa

As the discrepancy between the world's supply of protein and the growth of the global population continues

to widen, ways and means of bridging this gap have become a matter requiring immediate action. According to recent statistics (28), it can be estimated that there will be approximately six billion people on the face of the earth by the year 2000. In order to maintain the level of nutrition which we have at present, a two-fold increase in the protein supplied by plant materials and a four-fold increase in the protein of animal origin would be required. Experts (28) feel that it is unrealistic to expect such an increase in animal protein in most of the poorer countries because of the cost of production and their religious and cultural practices.

The simple fact remains that man and beast will ultimately be forced to compete for the same living space and the same available food supply (29). Thus, it is expected that plant proteins will emerge as the major source of dietary protein for the future. The production of cereal grains, wheat, corn and rice, could be expanded to provide the protein required. Cereal grains, however, are of poor quality because of an inherent deficiency of certain essential amino acids, particularly lysine and methionine. It is possible that the quality of the cereal

grains could be increased by breeding, such as was done in the case of high-lysine corn. On the other hand, the protein derived from oil-bearing seeds, such as soybean, cottonseed, and the peanut, or from legumes, such as alfalfa, peas and beans, are protein rich if somewhat deficient in the sulfur-containing amino acids. They could, however, be combined with cereal proteins to produce a protein mixture of high nutritive value (30). Such vegetable diets have been used successfully in child feeding programs (31,32) and have been used to cure kwashiorkor (33), the disease resulting from protein malnutrition. Cereal grains have a low level of protein content (6-14%), compared to the protein content of oilseeds (20-50%) and legumes (17-25%). This means that there is a greater protein yield per acre with oilseeds and legumes. This is of particular importance in countries where arable land is limited. Oilseeds and legumes can be cultivated in both the tropics and the temperate zones. At present, however, the oilseeds and legumes are grown in smaller quantities than the cereals, and they contribute only about 8% of the world's supply of protein. About 95% of the leguminous plants produced are fed directly to livestock at a

relative rate of 5 lbs plant protein for every 1 lb of animal protein produced (34).

The use of alfalfa as feed has been mainly limited to livestock. As a result, a great deal of research has been devoted to determining the chemical composition and feeding value of alfalfa (35,36,37). The data in Table I represent average values for the more important constituents of alfalfa (38). This table allows one to make certain generalization concerning the food value of the crop. The protein content is high, and the quality of protein is good except that the methionine content is low and may have to be supplemented with oilmeal products or synthetic methionine. Alfalfa is low in starch and sugars and relatively high in cellulose. Thus, if the energy requirements are high, it may have to be supplemented with cereal grains. Adequate amounts of minerals are usually present, if provision is made for certain areal deficiencies of phosphorous, cobalt, and iodine. Alfalfa is particularly rich in calcium, and, with the exception of B₁₂, it is also a rich source of vitamins--especially provitamin A (carotene). Even Vitamin D is supplied in fair quantities in sun-cured alfalfa hay or meal, or in wilted silage.

Table 1. Chemical Composition of Alfalfa Feedstuffs* (38)

Class	Analysis	Hay		Meal	
		All analyses	Early bloom	All analyses	Dehydrated
		Mean	Mean	Mean	Mean
Proximate Analysis	Dry matter--per cent	89.7	90.0	92.2	92.5
	Crude protein--per cent	17.3	18.4	18.2	18.9
	Ether extract--per cent	2.1	2.2	2.5	2.8
	Crude fibre--per cent	31.4	29.8	28.0	26.8
	Ash--per cent	8.9	9.4	10.3	10.4
	Nitrogen-free extract--per cent	40.3	40.2	41.0	41.1
Minerals	Calcium--per cent	1.64	1.25	1.35	1.19
	Phosphorus--per cent	0.26	0.23	0.30	0.32
	Copper--mg./lb.	6.2	6.1	8.5	9.6
	Potassium--per cent	1.77	2.08	2.46	2.86
	Magnesium--per cent	0.32	0.30	0.34	0.36
	Iron--per cent	0.24	0.20	0.049	0.065
	Manganese--mg./lb.	23.5	14.3	21.1	21.5
	Sulphur--per cent	0.36	0.30	0.27	
	Sodium--per cent	0.16	0.15	0.19	0.19
	Chlorine--per cent	0.28	0.38	0.74	0.72
	Cobalt--mg./lb.	0.060	0.043	0.105	0.093
	Zinc--mg./lb.	7.7		15.9	15.9
Iodine--meg./lb.	55				

Table 1 (continued)

Class	Analysis	Hay		Meal	
		All analyses	Early bloom	All analyses	Dehydrated
		Mean	Mean	Mean	Mean
Vitamins	Thiamin--mg./lb.	1.6		2.1	
	Riboflavin--mg./lb.	6.7	7.3	6.7	6.2
	Pantothenic acid--mg./lb.	11.7		17.4	
	Niacin--mg./lb.	18.2		16.5	
	Pyridoxine--mg./lb.				
	Choline--mg./lb.	693		547	
	Carotene--mg./lb.	27.7	57.7	47.4	36.2
	Folic acid--mg./lb.	1.54		2.86	2.48
	Biotin--mg./lb.	0.08		0.16	
	Vitamin D--I.U./lb.	585			
	Vitamin B ₁₂	0.0			
	α-tocopherol--mg./lb.	71.2		193.3	193.3
	Vitamin K--mg./lb.	11.2			
	Essential Amino-acids	Arginine--per cent	0.8		1.0
Histidine--per cent		0.4		0.3	0.3
Isoleucine--per cent		0.9		0.9	0.9
Leucine--per cent		1.3		1.4	1.4
Lysine--per cent		1.1		1.1	1.1
Phenylalanine--per cent		0.8		0.9	0.9
Threonine--per cent		0.9		0.8	0.8
Tryptophan--per cent		0.2		0.3	0.3
Valine--per cent		0.8		0.9	0.9
Methionine--per cent		0.2		0.2	0.2

Table 1. (continued)

Class	Analysis	Hay		Meal	
		All analyses	Early bloom	All analyses	Dehydrated
		Mean	Mean	Mean	Mean
	Energy--therms/lb.	2.00	2.04	2.07	
	Lignin--per cent	10.6	9.4	10.7	
	Cellulose--per cent	23.2	20.2	24.0	21.1
Energy and	Hemicellulose--per cent	8.6			
Carbohydrates	Total sugars--per cent	5.0	5.7	4.0	4.0
	Starch--per cent	2.2	1.8		
	Pentosans--per cent			13.4	13.4
	Pectins--per cent	5.6	5.6		

* All analyses are reported on a moisture-free basis (except for analysis of dry matter).

Alfalfa is considered high quality feed for beef and dairy cattle, sheep, horses, mules, swine, and poultry.

The human consumption of the young leaves of alfalfa as a vegetable has been reported in China (39). A few serious attempts have been made to encourage the human consumption of alfalfa in the United States. M. H. Haggart (40) obtained manufacturer's samples of a blended flour, "tea", and "coffee", made from alfalfa, together with samples of alfalfa cookies, crackers, breakfast food and candy. She states that the crackers, cookies, and breakfast food were palatable, but that the candy had such a pronounced alfalfa flavor that it was not relished.

Possibly the most extensive tests of alfalfa as a vegetable were made by Stharamesi and Falabella (41). They stressed the nutritive value of the leaves, and suggested using only tender leaves from the first crop harvested before the plants flower. Stems are avoided because of their high fibre content. These authors considered that 50 gms per day of alfalfa could be eaten easily, and used this quantity as a basis for the preparation of a number of dishes. Bolton (37) also has a number of recipes using alfalfa in his book.

It is also important to consider the negative nutritional aspects of the oilseeds and legumes. The oilseeds and legumes contain a wide variety of chemical substances which can exert undesirable effects when ingested by man and animals. The subject of naturally occurring toxicants in food has been reviewed by several authors (7,40,41). When alfalfa meal is fed to chicks in quantities above 5%, it may or may not act as a growth depressant. Research has shown that about 50% of chicks fed 10% alfalfa meal will suffer growth depression (42). As was the case with soybeans, part of the growth depression was due to the presence of saponins. In chicks fed raw soybean meal, hypertrophy of the pancreas has been noted (43,44). It has been suggested that the growth depression caused by the trypsin inhibitor may be the result of endogenous loss of essential amino acids derived from a hyperactive pancreas which is responding in a compensatory fashion (probably hormonally) to the effects of the trypsin inhibitor (44). It is not known if the trypsin inhibitor from alfalfa has the same effect on the pancreas as does the soybean trypsin inhibitor. Because man's dependence on plant protein can be expected to increase, it is

important to know the role and effect on animals of the naturally occurring toxicants. In order to better understand their nutritional effects, the chemical characteristics of the toxic plant components must be assessed. This knowledge will allow appropriate processing of oilseeds and legumes to remove or destroy constituents which could cause undesirable effects.

Current Status of Alfalfa Inhibitor Research

The presence of a trypsin inhibitor in the seeds of alfalfa was first reported by Borchers and Ackerson (45) in 1947. Kendall (46), in 1952, reported that prebloom alfalfa leaves also contained a trypsin inhibitor. Mitchell (47), in 1957, confirmed the presence of a trypsin inhibitor in alfalfa hay.

Mitchell (48) attempted isolation of the trypsin inhibitor from alfalfa hay in 1960. The alfalfa inhibitor was isolated using ethanol precipitation of an aqueous acidic extract. The amber colored precipitate showed the presence of two inhibitory components upon disc gel electrophoresis at pH 8.6. They concluded that the inhibitor was heat labile since 60% of the inhibitory activity was

destroyed within 30 minutes upon incubation of the isolated inhibitor in solution at 98°C. A Lineweaver-Burk double reciprocal plot indicated inhibition by the alfalfa inhibitor was noncompetitive.

Mooijman (49), in 1965, attempted isolation of the trypsin inhibitor from alfalfa hay using dialysis of acidic extracts with subsequent ion-exchange chromatography on DEAE cellulose. Upon dialysis, the inhibitory activity separated into two fractions. The non-dialyzable fraction inhibited trypsin only, while the dialyzable fraction inhibited both trypsin and chymotrypsin. All interactions were termed irreversible. Upon DEAE cellulose chromatography, the non-dialyzable fraction separated into four peaks containing trypsin inhibitory activity. All four peaks showed foam forming capacities, while three had hemolytic activity. This led him to suggest that the trypsin inhibition was possibly due to a saponin-peptide. The inhibitory material was stable between pH 2 and 12 and was demonstrated to be extremely thermostable.

Using ammonium sulfate precipitation and DEAE cellulose chromatography, Mitchell and Chien (50) in 1969 isolated an inhibitor fraction from alfalfa hay which showed

two bands upon electrophoresis (pH 8.6). Heating to 98°C for 30 minutes caused loss of about 50% of the inhibitory activity. A qualitative test by the method of Dubois (51) showed the presence of carbohydrate, suggesting to them that the inhibitor might be a glycoprotein, as are some trypsin inhibitor isolated from animal sources.

It is well established that a trypsin inhibitor is found in both the seeds and the vegetative portions of the alfalfa plant. Isolation of the inhibitor from the vegetative portions of the alfalfa plant has been attempted but has been unsuccessful. No attempt has been made to isolate the inhibitor from alfalfa seeds.

Characterization studies on the crude inhibitor preparations have shown foam forming capacity, hemolytic activity, and the presence of carbohydrate. The nature of the inhibition has been indicated to be both irreversible and noncompetitive. The heat stability of the inhibitor is in question as evidence has been presented to support both heat stability and lability. Suggestions have been made that the trypsin inhibition observed in alfalfa could be due to a saponin-peptide or to a glycoprotein, neither of which was isolated.

Research Objectives

The general objectives of this work was to detect the presence of, to isolate, and to characterize the protein trypsin inhibitor(s) present in the seeds of alfalfa (Medicago sativa). Specific objectives are listed below:

- a. to develop suitable extraction procedures resulting in the high yield solubilization of protein trypsin inhibitor with subsequent determination of its quantity.
- b. to develop high yield isolation procedures making use of affinity chromatography with insolubilized trypsin.
- c. to examine basic molecular characteristics of the trypsin inhibitor such as amino acid composition, molecular weight, terminal amino acid residues, stability toward various denaturants and binding characteristics.

MATERIALS AND METHODS

Enzyme Assays

Trypsin. The potentiometric determination of trypsin esterase activity was carried out by the method of Hummel (52). The synthetic substrate was tosyl arginine methyl ester (TAME). Ten milliliters of 5 mM TAME solution containing 50 mM CaCl_2 was placed in the jacketed incubation chamber of a Radiometer TTIA pH-stat, thermostatted at 25°C, and stirred under nitrogen. At zero time, about 0.01 mg of trypsin was added and the pH titrated to 8.0 using 0.05N NaOH. As hydrolysis proceeded, base was added to maintain the pH constant at 8.0. The chart recorder monitored the addition of base as a function of time. The rate of addition of base (slope) was an indication of the initial rate of hydrolysis.

Inhibitor solutions were assayed using assay cocktail consisting of 100 λ 0.1M TRIS·HCl pH 7.5, 100 λ inhibitor solution, and 20 λ of trypsin (1 mg/ml). This was allowed to incubate at room temperature for five minutes. Then, 100 λ was introduced into the TAME substrate solution and the initial rate of hydrolysis was determined from the rate of base addition.

One unit of enzyme activity was defined as that amount of trypsin which would hydrolyze one micromole of substrate per minute under the conditions described.

The Specific Enzyme Activity can be calculated from the initial rate of hydrolysis of TAME. The slope of the line when multiplied by the normality of the base gives the μM TAME hydrolyzed per minute. Subsequent division by the mg of trypsin used yields the Specific Enzyme Activity of trypsin in the assay.

$$\frac{\text{slope (ml/min)} \times (\text{N base})}{\text{mg TRP}} = \text{S.E.A.}$$

The difference between the specific enzyme activity for the inhibited and noninhibited trypsin assays allowed calculation of percent inhibition.

For example:

	N base = 0.05; mg trypsin = 0.0091			
	slope	SEA	Δ SEA	% inhibition
Trypsin only	.038	209		
Trypsin + inhibitor	.025	137	72	34%

The following examples of calculations are based on the above values.

The weight of inhibitor present in the test solution was calculated using a MW value of 7800 for the inhibitor

and 23,800 for trypsin. The molar binding ratio of the inhibitor trypsin complex was assumed to be 1:1, i.e.,
 (ug trypsin in assay mixture) (% inhibition) = ug trypsin inhibited.

For example:

from above:

ug trypsin in total assay mixture = 20 ug

% inhibition = 34%

$(20)(.34) = 6.8$ ug trypsin inhibited

The inhibitor present was calculated using a proportion involving molecular weights.

$$\frac{\text{ug trypsin inhibited}}{\text{trypsin molecular weight}} = \frac{X \text{ ug inhibitor (assay sample)}}{\text{inhibitor molecular weight}}$$

For example:

$$\frac{6.8 \text{ ug trypsin}}{23,800} = \frac{X \text{ ug inhibitor}}{7800}$$

$X = 2.21$ ug inhibitor (assay sample - (100λ))

This value gives the ug inhibitor present in one assay. In order to determine how much is present in the total preparation one must multiply by the total volume of inhibitor solution, i.e., (ug inhibitor/assay volume) (total sample vol) = ug inhibitor (total sample).

For example:

(assume 500 mls inhibitor solution)

$2.21 \text{ ug}/0.1 \text{ ml} \times 500 \text{ ml} = 11.05 \text{ mg inhibitor (total sample)}$

Chymotrypsin. Chymotryptic esterase activity was determined using the potentiometric method of Walsh and Wilcox (53). Assay procedures are the same as those described for trypsin except that the esterolytic substrate was N-acetyl-L-tyrosine ethyl ester (0.01 M).

Elastase. The potentiometric assay of Kaplan and Dugas (54) using the synthetic substrate, N-benzoyl-L-alanine methyl ester, was used to determine the activity of elastase. The assay procedure was the same as that of trypsin.

Carboxypeptidase A. N-benzoylglycyl-L-phenylalanine (hippuryl-L-phenylalanine) was used as substrate for the spectrophotometric assay system of Folk and Schirmer (55). The measurements to detect inhibitor activity were carried out at 25°C in 1 cm cells in a Varian Techtron 635 recording spectrophotometer at 254 nm.

Carboxypeptidase B. The assay used was similar to that of CPA. The substrate was N-benzoylglycyl-L-arginine (hippuryl-L-arginine) (56).

Pepsin. Pepsin activity was assayed according to the method of Anson (57). Acid-denatured hemoglobin was mixed with pepsin at pH 1.8 and 37°C and the release of cleavage products soluble in 3% TCA was measured spectrophotometrically at 280 nm.

Isolation Procedure

Seeds of alfalfa (Medicago sativa), variety Ladock 65, were ground dry in a CRC micro-mill for two minutes producing a fine powder. The ground meal was defatted in a Soxhlet apparatus using a chloroform:methanol mixture (2:1 vol:vol) for 24 hours. The defatted meal was air dried and reground to a fine powder with a mortar and pestle and stored in an amber bottle at room temperature until use.

The weighed meal was extracted in a solution containing 0.05M ascorbic acid, 0.1M NaCl and 30% (vol) ethanol at pH 4.0. The mixture (1gm:5mls) was stirred at room temperature for varying lengths of time. After

extraction, solid debris was removed by filtration through a double layer of cheesecloth and the resulting supernatant clarified by centrifugation at 12,500 rpm for 20 minutes in a Sorval RC2B centrifuge. The pH of the supernatant liquid was adjusted to pH 7.5 using 6N NaOH and re-centrifuged at 12,500 rpm for 10 minutes.

Affinity Chromatography

Preparations of trypsin-sepharose. Attachment of trypsin to the insoluble support was based on the method described by Cuatrecasas (58). Sepharose 4B (Pharmacia) activation was carried out in a 250 ml beaker containing a pH electrode, thermometer, and a magnetic stir bar. After washing on a sintered glass filter to remove azide, the damp resin (60 ml) was suspended in 30 ml of water and 4.40 gm cyanogen bromide (dissolved in 25 ml of water) was added. The pH was rapidly adjusted to 11.0 and maintained at that pH with additions of 6N NaOH. The temperature of the solution was maintained at 20°C by the periodic addition of ice. After 14 minutes the reaction ceased and the mixture was immediately filtered by suction on a sintered glass filter and washed quickly with 2 liters of ice water.

and 1 liter of cold 0.05M sodium borate buffer, pH 9.1. Filtration and washing of the activated sepharose took less than 5 minutes.

One gm of trypsin was dissolved at 4°C in 25 ml of 1.2 mM HCl containing 0.01M CaCl₂. Twenty-five ml of 0.05M sodium borate buffer, pH 9.1, was added to the trypsin solution immediately before addition of the activated sepharose. The pH was adjusted to 9.1 with 3M NaOH and the mixture was stirred gently on a magnetic stirrer at 4°C for 20 hours.

After coupling, the trypsin-sepharose was filtered by suction on a sintered glass filter and washed with 3 liters of a solution containing 0.05M sodium borate, 0.5M NaCl, and 0.01M CaCl₂ at pH 9.1. The insolubilized trypsin preparation was stored at 4°C in a small amount of 1.2 mM HCl containing 0.01 CaCl₂.

Absorption of inhibitor. The sepharose-trypsin resin was added to the clarified alfalfa supernatant at room temperature and allowed to stir at pH 7.5 for 20 minutes. The deeply colored resin was collected by suction filtration and washed with a 0.01M TRIS·HCl buffer pH 7.5

containing 0.1M NaCl and 0.01M CaCl₂ until the OD₂₈₀ of the effluent was less than 0.05 absorbancy units.

Dissociation of inhibitor:trypsin-sepharose complex.

A low pH buffer, 0.1M β-alanine, 0.1 NaCl, pH 2.1 was added to the sepharose-trypsin resin in batch fashion and allowed to stir. After 10 minutes, the buffer containing the inhibitor was removed from the sepharose by suction filtration. The pH of the solution was raised to 7.5 and concentrated using an amincon pressure cell fitted with a UM-05 ultrafiltration membrane using 75 psi nitrogen pressure. The concentrate was desalted using Sephadex G-10, G-50 or Bio-gel P-2 and subsequently lyophilized.

Chromatography

Molecular-sieve. Columns of Sephadex G-10, G-50 and Bio-gel P-2 were prepared according to the method first illustrated by Lathe and Ruthven (59). Fully hydrated gel particles were decanted several times to remove the fines and degassed by placing under vacuum until evolution of dissolved air ceased (10-15 minutes). Packing was done at room temperature by filling the column partially with buffer solution, into which a portion of the gel

slurry was poured. Another column was placed on top and the entire gel slurry was introduced and allowed to settle under gravity. After settling the column was pumped with the appropriate buffer until approximately two bed volumes had passed through the column.

Ion exchange. Carboxymethyl cellulose (Whitman type CM 52) was washed repeatedly with 0.5M NaOH, with filtration on a Bucher funnel between washings, to remove colored material. Excess alkali was removed by washing with water. The resin was resuspended in 0.02 sodium acetate pH 5.0 prior to column preparation. A column 2.2x25 cm was poured and allowed to settle under gravity. The flow rate was maintained at 12 mls/hr. by use of a Milton Roy piston pump. Sample elution was accomplished using a linear salt gradient consisting of 0.02M sodium acetate pH 5.0 initial and 0.2M sodium acetate pH 5.0 final buffers. Rechromatography was accomplished using a linear salt gradient consisting of 0.05M sodium acetate pH 5.0 initial and 0.15M sodium acetate pH 5.0 final buffers.

Sample loading. Samples in a small volume of elution buffer were applied to the column and allowed to sink into the top of the bed under gravity, followed by a small volume buffer wash. A layer of buffer a few centimeters deep was placed over the top of the bed, the column was then connected to a reservoir or pump and elution started. The effluent absorbance was monitored by directing it through a Gilford single beam spectrophotometer. The effluent was subsequently collected using a fraction collector.

Binding Studies Using p-nitrophenyl-p-guanidinobenzoate-HCl

The sodium veronal-HCl buffer and the NPGB solution were prepared according to Chase and Shaw (60) and filtered through millipore filters before use. Trypsin was dissolved in cold 1 mM HCl containing 0.02M CaCl₂ to a final concentration of 10 mg/ml. Alfalfa inhibitor was dissolved in 0.01M TRIS·HCl, pH 8.0. Both protein solutions were centrifuged to remove any insoluble material.

The assay conditions were modified from the Chase and Shaw procedure to allow addition of different volumes of trypsin inhibitor. The final volume of the total cocktail always equalled 1 ml with a final pH of 8.30.

In a typical experiment, inhibitor and trypsin were allowed to equilibrate for 5 minutes before NPGB was added. Corrections for nonenzymatic hydrolysis were obtained from separate control runs without trypsin. Assays were run at instrument room temperature ($20 \pm 1^\circ$) and the cells were not thermostatted. A Cary model 14 spectrophotometer equipped with the 0 to 0.1 absorbance slide wire was used. Absorption cells were 1 cm path length. All calculations were done using a E_{410}^M (pH 8.30) for p-nitrophenol of 16,370.

Heat Stability

Alfalfa inhibitor (0.05 mg) was dissolved in 3 ml of 10 mM TRIS·HCl, 0.50 mM NaCl at pH 7.5 and incubated for varying lengths of time in a constant temperature bath. Aliquots were taken at timed intervals and assayed to determine inhibitory activity remaining.

Carbohydrate Content

The qualitative carbohydrate test of Dubois (51) was used. Purified inhibitor and sugar standards were treated with 0.05 ml of 89% phenol. After mixing, 0.5 ml concentrated sulfuric acid was added and solutions were

placed in a 30°C water bath for 10 minutes. Absorbance was measured at 480 nm to test for the presence of pentoses and uronic acids and at 490 nm to test for the presence of hexoses.

Physical Characterization

Amino acid analysis. Approximately 1 mg samples of protein were hydrolyzed for 24 or 48 hours at 110°C in constant boiling HCl in sealed, evacuated tubes. Analysis was carried out on a Beckman-Spinco 120C amino acid analyzer, by the method of Spackman et al. (61). Data was collected automatically with use of an Infotronics CRS110A digital integrator.

Absorption spectrum. The ultraviolet spectrum of the alfalfa inhibitor in water was determined using a Cary model 14 spectrophotometer.

Electrophoresis. Basic (pH 8.3) and acidic (pH 4.3) disc gel electrophoresis was performed in 15% polyacrylamide gels as described by Davis (62). Protein samples (150-300 µg) containing sucrose were applied to the top of the gel and a current of 2mA/per tube was applied for

2 hrs. After each run the bands were visualized with 50% trichloroacetic acid and their positions recorded immediately.

Isoelectric focusing was done using LKB Produker ampholine mixtures (pH 3-10 and pH 7-10) in polyacrylamide analytical disc gel columns. The effect of urea on the system was studied by polymerizing the gel in 1,2,3, and 4M urea solutions. Tubes containing the gel-ampholine columns were placed in the electrophoresis tank with 0.2% sulphuric acid in the anodic compartment and 1% ethanola- mine in the cathode compartment. A current of 1mA/gel was maintained by slowly increasing the voltage to a maximum of 350 V. Complete focusing required from 2-4 hours. Visualization was accomplished with 50% TCA and up to 75% TCA with the urea gels. A sample of myoglobin was normally included in a separate tube in order to visually determine when focusing was completed.

Molecular weight determination. A 1 x 1000 cm col- umn of Sephadex G-50 (fine grade) was prepared as previ- ously described. Samples of marker proteins (1 mg) were dissolved in 0.5 ml of 0.05 TRIS·HCl, 0.1N NaCl at pH 7.5

and put on the column as previously described. The flow rate was maintained at 12 ml/hr and five minute fractions (2 ml) were collected.

Pepsin digest. An alfalfa inhibitor solution (0.075 mg) was incubated with a pepsin solution (0.006 mg) in a water bath at 37°C. Residual inhibitory activity was determined using a TAME assay.

Carboxypeptidase A and B digestion. Alfalfa inhibitor (0.7 mg) was dissolved in 0.3 ml of 0.25M TRIS·HCl, 0.1N NaOH (pH 8.3). CPA or CPB solution was added (substrate:enzyme 50:1 wt/wt) and allowed to react for 3 hours at 37°C. The reaction was stopped with the addition of 0.5 ml of 0.2N sodium citrate (pH 2.2) buffer. The entire sample was analyzed on the amino acid analyzer.

Chemical Modification

Dansylation. A small amount (1 µg) of alfalfa inhibitor was dissolved in 30λ of 0.01M sodium bicarbonate pH 8.5 and 30λ of DNS-Cl (2.5 mg/ml in acetone) was added. Reaction continued overnight in the dark at room temperature. After drying, 1 ml of constant boiling HCl was

added and the sample was hydrolyzed for 18 hours at 110°C in sealed evacuated tubes. The dried hydrolyzate was dissolved in acetone and spotted on thin layer plates (Beckman nonfluorescent). Two solvent systems were used:

1. Chloroform, t-butyl alcohol, acetic acid (6:3:1);
2. Chloroform, benzyl alcohol, acetic acid (70:30:3).

Reduction and alkylation. Alfalfa inhibitor (7 mg) was dissolved in 1 ml of 6M guanidine·HCl and 0.01M Na₂CO₃ pH 8.0. The solution was bubbled with N₂ before and after addition of protein. Reduction was allowed to proceed overnight after addition of a 10 fold excess (over disulfide bonds) of dithiothreitol (63) at room temperature under N₂. Alkylation was accomplished by the addition of a 10 fold excess (over dithiothreitol) of iodoacetamide (recrystallized from ethyl ether) dissolved in a 1 ml of 3M TRIS·HCl (pH 8.0). The reaction was quenched in 10 minutes by addition of excess β-mercaptoethanol and the total mixture was placed on a Sephadex G-50 column and eluted with H₂O. The eluted material was detected by absorbancy at 230 nm, collected and lyophilized.

Carbamylation. Amino terminal residues of the alfalfa inhibitor and the reduced and alkylated material were determined using the cyanate method of Stark (64). Alfalfa inhibitor (2.1 mg) and the reduced and alkylated (1.6 mg) alfalfa inhibitor were dissolved in 0.1 ml of 0.2M N-ethylmorpholine acetate buffer pH 8.0. One hundred mg of KOCN had previously been added to 2 ml of the buffer. Carbamylation was carried out at 50°C for 18 hr. Excess cyanate was then destroyed by the addition of glacial acetic acid and the sample was dried by evaporation. After cyclization, the hydantoins were collected and hydrolyzed in 0.2N NaOH. The hydrolyzate containing the amino acids was neutralized and applied to the amino acid analyzer.

Reagents

Sources of reagents are listed below. All other chemicals were of analytical grade. Water was distilled and deionized on a column of Barnstead research model deionizer.

<u>Enzyme</u>	<u>Manufacturer</u>	<u>Type or Code</u>
Trypsin	Sigma and Worthington	TRL IIA
Chymotrypsin	Worthington	CDI OBK
Elastase	Sigma	E-0127
Carboxypeptidase A	Worthington	COBC 7LA
Carboxypeptidase B	Worthington	COBC 3AA
Pepsin	Sigma	P-6875
Ovomucoid	Worthington	OI ICA
N-tosyl-L-arginine methyl ester	Nutritional Biochemicals Co.	
N-acetyl-L-tyrosine ethyl ester	Gift by Dr. J. C. Powers	
N-benzoyl-L-alanine methyl ester	Sigma	
N-benzoylglycyl-L-phenylalanine	Schwarz-Mann	
N-benzoyglycyl-L-arginine	Schwarz-Mann	
hemoglobin (substrate)	Nutritional Biochemicals Co.	
dithiothreitol	Calbiochem	
p-nitrophenyl-p-guanidinobenzoate-HCl	Nutritional Biochem- icals Co.	
p-nitrophenol	Worthington	
iodoacetamide	Aldrich	
β -mercaptoethanol	Aldrich	

RESULTS AND DISCUSSION

Extraction of the Alfalfa Trypsin Inhibitor from Ground Defatted Seeds

Detection of inhibitory activity. Figure 1 shows the inhibition curves obtained when 9.1 μg of chymotrypsin or trypsin was incubated with aliquots of alfalfa extract. The crude extract mixture consisted of 1 gm of ground defatted seeds to which was added 5 ml of mM HCl. The enzymatic activity of trypsin was inhibited more than 80%, while that of chymotrypsin was only inhibited 5%. The apparent concentration of the protein trypsin inhibitor was 0.4 mg/gm of defatted alfalfa seeds. This value was later adjusted downward after isolation of a non-proteinaceous low molecular inhibitory substance. In view of the preponderant amount of trypsin inhibitor in the alfalfa seed, the minimal chymotrypsin inhibitory activity was not investigated.

Factors affecting solubilization of the inhibitor.

Optimum conditions for solubilization of the trypsin inhibitor in alfalfa was determined by varying the pH and temperature of the extraction solvents, as shown in Table 2. The alfalfa inhibitor was solubilized more readily in acidic buffer. As the pH of the extraction buffer became

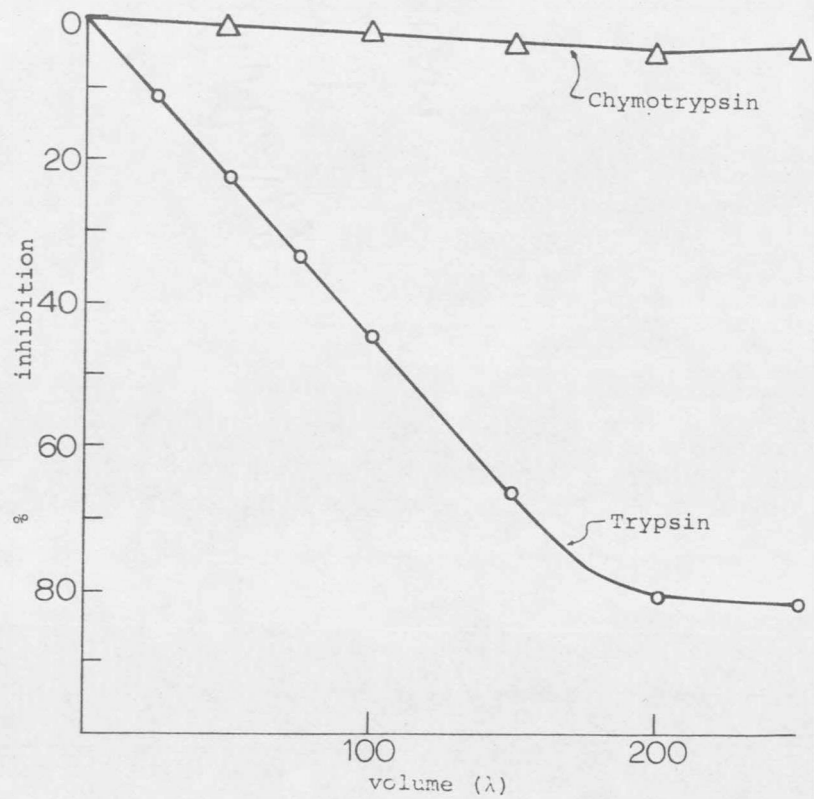


Figure 1. Inhibition of trypsin or chymotrypsin in the presence of alfalfa extract. Inhibition determined by TAME (trypsin) and ATEE (chymotrypsin) assay.

more alkaline, the amount of inhibitor in the supernatant steadily declined. Solubilization of the inhibitor also appeared to be temperature dependent, but not in an easily understandable way. As the extraction temperatures increased above 22°C, a steady decline in the supernatant inhibitor concentration was noted. This generalization does not apply at pH 7.0, where the concentration of supernatant inhibitor increased with higher temperatures. The highest concentration of alfalfa inhibitor was obtained using the pH 4.0 solution and 22°C.

Table 2. Effects of varying pH and temperature on the rate of solubilization of the alfalfa inhibitor*

Temperature (°C)	pH 4.0	pH 7.0	pH 9.0	pH 12.0
	% inhibition	% inhibition	% inhibition	% inhibition
7	25	9	9	17
22	29	13	8	7
47	21	15	10	12
71	15	17	8	3

* - extracts assayed after one hour using TAME assay

Figure 2 shows the increase in inhibitor concentration in the extract supernatant as a function of time.

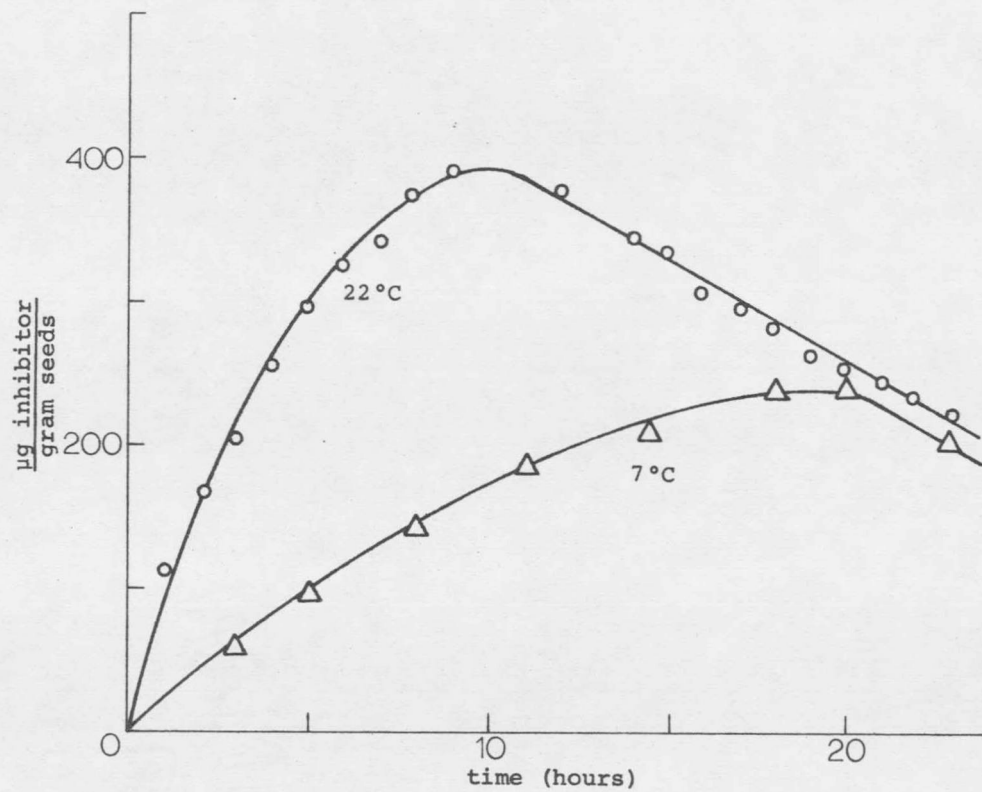


Figure 2. Appearance of trypsin inhibitory activity in extracts of alfalfa seeds.

At 7°C, the inhibitor concentration in the supernatant slowly increased, peaking at 18 hours with a concentration of 25 mg inhibitor/100 gm alfalfa seeds. At 22°C, the concentration of the inhibitor was 40 mg/100 gm seeds after 10 hours. For preparative purposes, extracts of ground defatted alfalfa seeds were performed using pH 4.0 solvents and 22°C for 10 hours.

After 10 hours, the concentration of the inhibitor slowly declined (Figure 2). The slow loss of activity observed could have been due to the action of endogenous serine proteinases, oxidation or slow destruction of susceptible inhibitory components, or to the presence of unstable non-protein inhibitors. Degradation of the inhibitor by endogenous serine proteinases was unlikely in that diisopropylphosphofluoridate (DFP), an irreversible serine proteinase inhibitor, did not retard the loss of activity. Possible oxidation of the inhibitor was likewise rejected when the use of ascorbic acid, a sacrificial oxidant and/or extraction under a nitrogen atmosphere did not retard the loss of activity. The presence in alfalfa seeds of an unstable, low molecular weight, non-protein inhibitor was detected in gel filtration experiments (below). This

material was likely responsible for the slow decline of inhibitory activity observed in the extracts.

Removal of solid debris from the extract by filtration through cheesecloth resulted in a very syrupy supernatant. The addition of ethanol (60% by vol) caused precipitation of fibrous material with a concurrent decrease in solution viscosity. Some loss of inhibitory activity was also noted. This loss was likely due to co-precipitation since the inhibitor was later observed to be soluble in high concentrations of ethanol. The use of 30% ethanol in extraction buffers reduced solubilization of the fibrous material and consequently reduced the syrupiness. Use of higher concentrations of ethanol would have caused possible inactivation of the insolubilized trypsin used later in the isolation procedure. Extraction buffers were therefore routinely made 30% in ethanol.

Amount of trypsin inhibitor present in alfalfa.

The total amount of trypsin inhibitor present in alfalfa was calculated using an inhibitor molecular weight of 7800 and an inhibitor-trypsin binding stoichiometry of 1:1. An example of this calculation was given in Materials and Methods. Ground defatted alfalfa seeds were found to

contain 40 mg of trypsin inhibitor per 100 gm of seed or 0.04% by weight (of defatted seeds). Using a value of 20% (by weight) protein in alfalfa seeds (37), the alfalfa inhibitor accounted for 0.2% of the total protein in the seeds. This compares with 6.0% of the total protein in soybeans present as trypsin inhibitors (65) and 0.11% present as trypsin inhibitors in sainfoin (66).

Isolation of Alfalfa Trypsin Inhibitor

Insoluble trypsin-sepharose preparation. The trypsin-sepharose was prepared using the method of Cuatrecasas (58). Before addition of the trypsin to the activated sepharose, the active site titrant, p-nitrophenyl-p'-guanidinobenzoate (NPGB) was used to determine the concentration of functional trypsin molecules. This method is based on the rapid and stoichiometric acylation of active trypsin by NPGB as measured by the consequent burst or liberation of p-nitrophenol. About 60% of the trypsin added to the activated sepharose was catalytically active. The coupling reaction was allowed to proceed overnight, after which high salt washes (0.5M NaCl) were used to remove any noncovalently bound trypsin. The A_{280} of these washes corresponded to 53.1 mg

of trypsin based on a $E_{280}^{1\%}$ for trypsin of 15.4 (67). Therefore, ninety-five percent of the trypsin added was covalently bound to the beads yielding 15.7 mg trypsin bound per ml of sepharose beads. After coupling, 43.5% of the formerly active trypsin molecules were still capable of reaction (acylation) with NPGB. A TAME assay indicated that after coupling only 2.4% of the formerly active trypsin molecules were capable of deacylation as measured by the production of substrate carboxyl groups.

Tryptic hydrolysis of substrate proceeds through two major steps, acylation and deacylation. Upon substrate bond cleavage, the serine hydroxyl (#189) of trypsin forms an acyl intermediate with the carboxyl portion of the substrate. Deacylation is then accomplished by a displacement reaction involving an activated water molecule. The deacylation step also involves a flexion of the amino acid backbone in the vicinity of the active site (68).

The sepharose bound trypsin appeared to be able to undergo acylation (NPGB), but was restricted in its capacity to deacylate (TAME) at neutral pH. Dissociation of a bound inhibitor from sepharose-trypsin would not be dependent on the ability of trypsin to deacylate since formation

of an inhibitor-trypsin acyl complex is not prerequisite for binding (25). This material should, therefore, in spite of its low TAME activity, be useful for affinity chromatography. In fact, the formation of an acyl intermediate with subsequent deacylation was not desirable because of possible consequent modification of the inhibitor molecule.

The capacity of the sepharose-trypsin to bind inhibitor molecules was determined using chicken ovomucoid. Chicken ovomucoid was chosen because of its large size (MW 29,000) which could be expected to maximize any accessibility problems encountered in binding to the sepharose-trypsin (compared to the smaller inhibitor). The concentration of ovomucoid was determined spectrophotometrically using an $E_{277}^{1\%}$ of 4.10 (69). Incubation of ovomucoid with sepharose trypsin revealed that 46% of the formerly active trypsin molecules bound inhibitor. Dissociation of the complex in low pH buffer confirmed the amount of inhibitor bound. This preparation of sepharose-trypsin was therefore useful for affinity chromatography because of its high degree of availability for inhibitor binding. Using the figures above, 1 ml of sepharose-trypsin beads could be expected to bind 1.75 mg of alfalfa inhibitor.

Adsorption of alfalfa inhibitor on to trypsin-sepharose. Extracts of alfalfa seeds were clarified by centrifugation. The pH of the amber colored supernatant was raised to 7.5 with 6N NaOH, causing the precipitation of a non-inhibitory material which was removed by centrifugation. Addition of sepharose-trypsin to the supernatant resulted in adsorption of nearly all inhibitory activity within 20 minutes. A small amount of residual inhibitory activity (5-7%) always remained in the supernatant. Repeated treatment with sepharose-trypsin did not remove it, indicating that the inhibition was due to a material which did not have a high affinity for trypsin or sepharose.

Removal of the sepharose-trypsin trypsin inhibitor complex from the extract supernatant was accomplished using suction filtration. The filtered beads were stained an amber color which could not be removed by repeated washing with Tris buffers containing 0.5M NaCl. The colored material could be removed from the sepharose beads by lowering the pH to 5.0. This, however, also removed some bound inhibitor. The colored material was determined by TAME assay to lack trypsin inhibitory activity. Birk (23), in

work on the soybean inhibitor, observed that some inhibition of trypsin was due to non-specific binding by saponins. The fact that the colored material was non-inhibitory suggested that it might be binding to the sepharose matrix rather than to the insolubilized trypsin. Birk had suggested that pre-incubation of the supernatant containing the non-specific material with another protein would scavenge it from the solution. If non-specific binding to either trypsin or the sepharose matrix was occurring, pre-incubation with serine albumin and free sepharose could be expected to immobilize the colored material. This was attempted at neutral pH. It was ineffective in removing the colored material adsorbed to the sepharose-trypsin beads. Selective removal of the colored material from the sepharose-trypsin trypsin inhibitor complex might have been possible by subjecting it to pH gradient elution. This procedure was not pursued because other researchers have found that lowering the pH slowly can result in trypsin cleavage of susceptible bonds in inhibitors (70).

Dissociation of sepharose-trypsin alfalfa inhibitor complex. The dissociation constant of other trypsin inhibitors have been shown to be pH dependent (71). Maximum binding occurs in the neutral pH range and as the pH is lowered, the dissociation constant increases until at pH 2.0 no effective binding takes place. Dissociation of sepharose-trypsin alfalfa inhibitor complex was accomplished by taking advantage of this pH dependence. Figure 3 shows the dissociation of the complex using 1 mM HCl. As the pH decreased, one symmetrical peak of inhibitor activity was eluted coincident with the decrease in pH.

It has been reported that dissociation of trypsin inhibitors from trypsin could result in production of artifacts if the pH drop was slow. Artifacts may result from cleavage by trypsin of a susceptible peptide bond in the inhibitor. The possibility of artifacts was investigated using a slow decline (elution with 1 mM HCl) and a rapid decline (elution with 0.2M β -alanine pH 2.1) in pH to dissociate the complex. The use of these acids, in both batch and column methods, yielded the same results as judged by disc gel electrophoresis. The method of elution from sepharose-trypsin does not change the product obtained in the case of the alfalfa inhibitor.

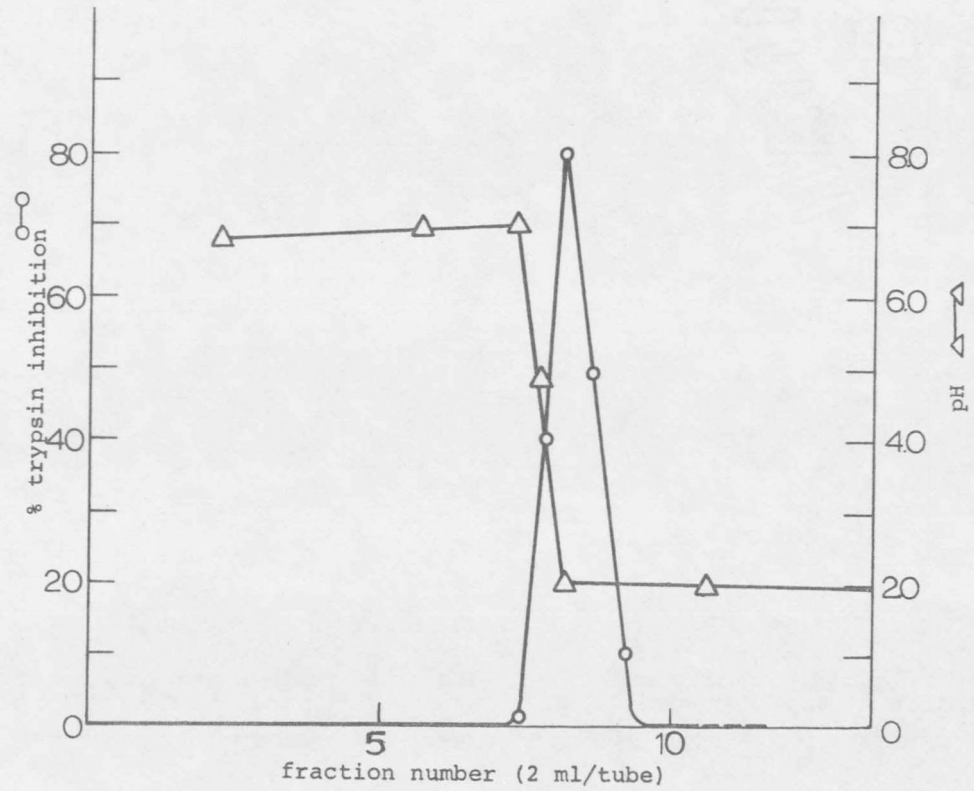


Figure 3. Removal of alfalfa trypsin inhibitor from sepharose-trypsin by column elution with pH 2.1 β -alanine buffer.

The solution containing the inhibitor was neutralized with 6N NaOH and concentrated over a UM-05 ultrafiltration membrane. The concentrate was desalted on a G-50 Sephadex column in water and lyophilized. The result was a white fluffy powder which was used for further experimentation.

Characterization of the Alfalfa Trypsin Inhibitor

Gel filtration. After the inhibitor solution was concentrated in the UM-05 ultrafiltration cell, it was subjected to gel filtration on Sephadex G-50 as shown in Figure 4. The elution pattern was followed spectrophotometrically at 230 nm because the inhibitor had little adsorbance at 280 nm. Three peaks eluted from the column, two of which contained inhibitory activity. The major peak normally contained 60% of the inhibitory activity from the initial crude extract. A non-inhibitory peak on the column void contained all of the amber color. Twenty-seven percent of the initial inhibitory activity was found in the low molecular weight peak. This peak was concentrated, refiltered on G-10 and lyophilized resulting in an off-white solid material. Amino acid analyses, after hydrolysis, indicated the absence of significant amounts of

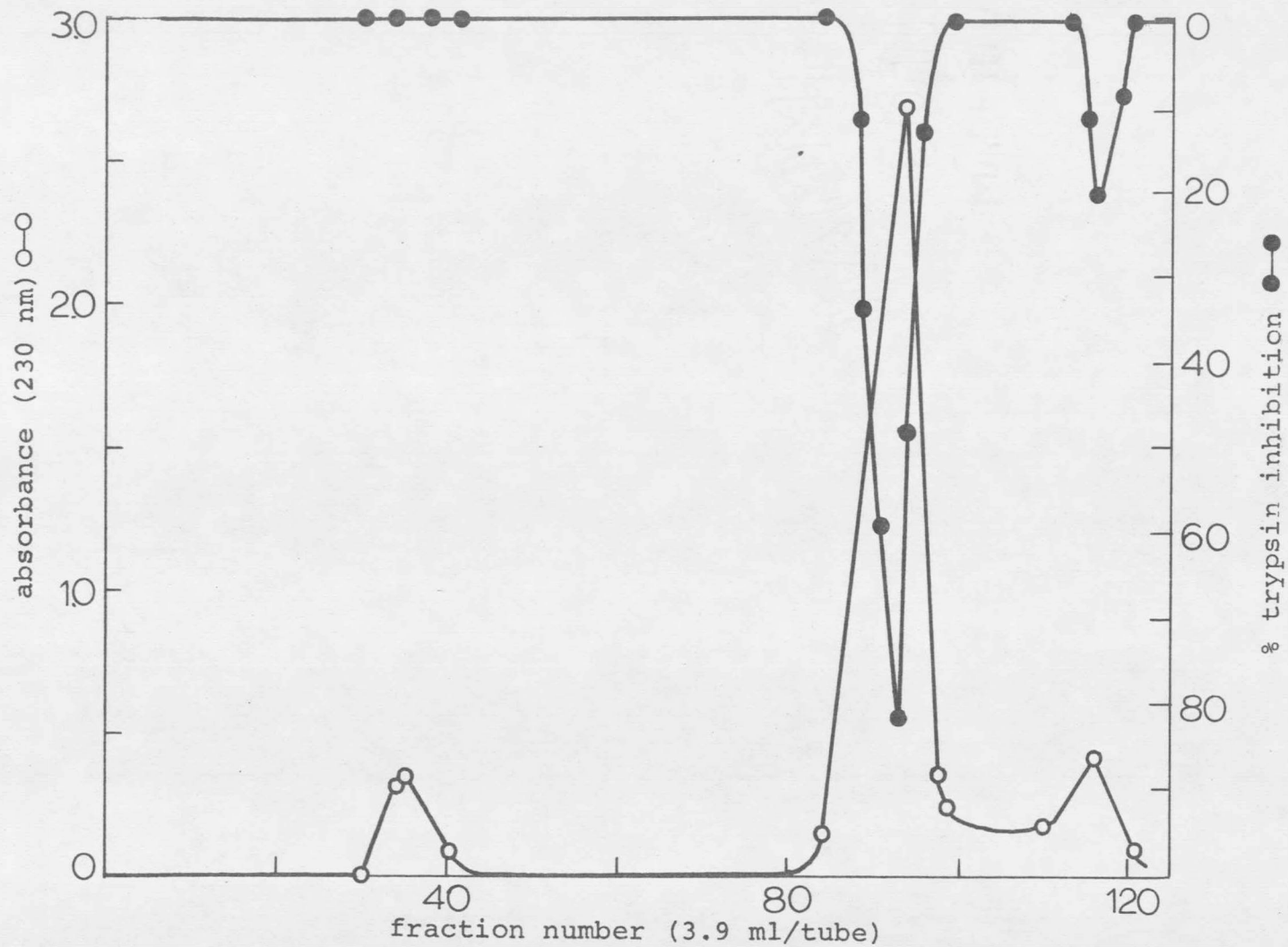


Figure 4. Sephadex G-50 gel filtration in water of alfalfa inhibitor.

amino acids. Inhibition by this material appeared to be specific for trypsin since pre-incubation of the peak with serum albumin did not remove any inhibitory activity. The inhibitory activity contained in the low molecular weight peak decreased slowly with time, suggesting that it could have been responsible for the decline in inhibitory activity observed in alfalfa seed extracts. It appeared that some of the inhibitory activity observed in the initial extract was due to this non-protein constituent. This meant that only 0.13% of the protein content of alfalfa seeds was present as protein trypsin inhibitor. The major peak from gel filtration contained most of the inhibitory activity that could be associated with protein.

Amino acid composition. Table 3 shows the amino acid composition of the major inhibitory peak isolated from alfalfa seeds using G-50 gel filtration. The alfalfa inhibitor contained approximately 58 amino acid residues indicating an approximate molecular weight of 6380. This analysis was based on the seemingly constant presence of one histidine. An assumption was made that all the inhibitor molecules present contain only one histidine. If

some contain no histidine, the value determined for the molecular weight would be low. The inhibitor contained no methionine, tryptophan and only trace amounts of valine. Half cystine (14 residues/molecule) accounted for 23 percent (by weight) of the molecule. This, along with the high proline content, was suggestive of a compact structure. The absence of methionine and tryptophan along with large amounts of half cystine and proline is characteristic of proteinase inhibitors isolated from legumes (72).

Table 3. Amino acid composition of alfalfa inhibitor isolated using Sephadex G-50*

Amino acid		Amino acid	
Lysine	2.42	Alanine	2.72
Histidine	1.00	Cystine	14.11
Arginine	3.88	Valine	0.83
Aspartic acid	5.58	Methionine	0
Threonine	9.34	Isoleucine	4.16
Serine	5.38	Leucine	1.06
Glutamic acid	3.46	Tyrosine	0.80
Proline	6.20	Phenylalanine	<u>2.01</u>

58

Approximate molecular weight	6380
Total Recovery Protein	67%

* - based on one histidine

Ultraviolet absorption spectrum. Figure 5 shows the molecular absorption spectrum of the alfalfa inhibitor ($3.8 \times 10^{-4} \text{ M}$) which was done in water. The absence of tryptophan in the alfalfa inhibitor suggested by amino acid analysis was confirmed as the spectrum lacks a tryptophan shoulder at 292 nm. The spectrum showed the presence of some tyrosine as indicated by the absorbancy noted at 280 nm. The lack of an absorption trough in the 250 nm region was likely due to the presence of large amounts of half cystine (73). A molar extinction coefficient was calculated from the absorbancy value at 280 nm.

The extinction coefficient was calculated using the Beer-Lambert equation.

$$(1) \quad A_{280} = E_{280}^M \quad bc$$

A = absorbancy (280 nm)

E_{280}^M = molar extinction coefficient (280 nm)

b = cell length (cm)

c = concentration (M)

$$0.44 = E_{280}^M \quad (1 \text{ cm}) (3.8 \times 10^{-4} \text{ M})$$

$$E_{280}^M = \frac{0.44}{3.8 \times 10^{-4} \text{ M cm}} = 1150 \text{ M}^{-1} \text{ cm}^{-1}$$

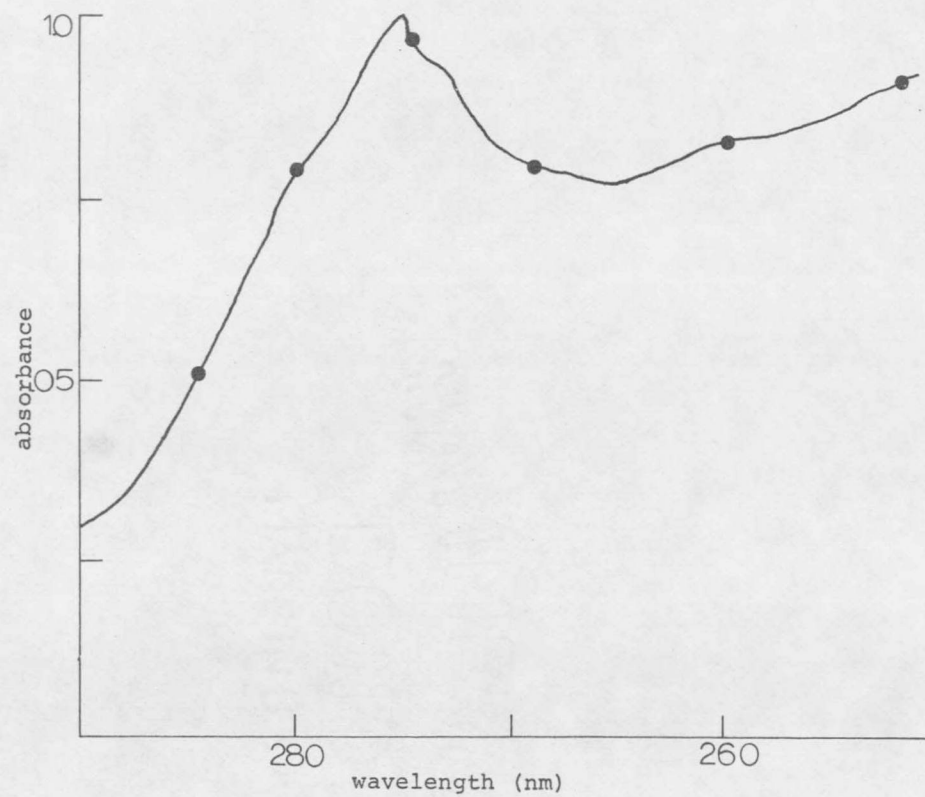


Figure 5. Molecular absorption spectrum in H_2O of the alfalfa trypsin inhibitor.

The molar extinction coefficient calculated was $1150\text{M}^{-1}\text{cm}^{-1}$. This calculated value corresponded to the presence of one tyrosine which amino acid analysis had also indicated. If any tryptophan would have been present, the calculated extinction coefficient would have been much larger. The calculated value indicates that one tyrosine was present per mole of alfalfa inhibitor and supported the lack of tryptophan.

Disc gel electrophoresis. When the alfalfa inhibitor was subjected to pH 4.3 disc gel electrophoresis, only one protein band was observed. Visualization of the protein band was accomplished by soaking the gels in a 50% trichloroacetic acid solution. The protein precipitated readily but redissolved in less than one hour. The precipitated band was also easily redissolved in neutral pH buffer with retention of inhibitory activity. Ion exchange chromatography of the same material indicated the presence of at least three inhibitory species. The possibility that the single band might be two or more closely spaced bands was investigated by varying the protein load on the gels. With protein loads between 150-300 μg , no visible change in the shape of the single band was observed. Since

the acidic disc gel electrophoretic data didn't reflect the apparent heterogeneity observed on ion exchange chromatography, other tests of purity were undertaken.

Isoelectric focusing. Analytical isoelectric focusing using carrier ampholytes was chosen because of the technique's extraordinary resolving power (73). Figure 6 shows the results of electrofocusing the alfalfa inhibitor in a pH 3-10 ampholyte gradient. Upon visualization in 50% trichloroacetic acid, it was observed that the material had separated into two heavy and one light bands. A colored protein, seal myoglobin, was normally focused in a separate gel to visually indicate when focusing was completed. The two heavy bands of alfalfa inhibitor focused above the major band of seal myoglobin (pH = 8.34) indicating isoelectric points more basic than that of myoglobin. The light band focused slightly below the major band of seal myoglobin. All three precipitated protein bands were eluted from the gel at neutral pH and were found to contain inhibitory activity. These results were supportive of the apparent heterogeneity observed on ion exchange chromatography and suggested a possible separation method based on differences in isoelectric pH.

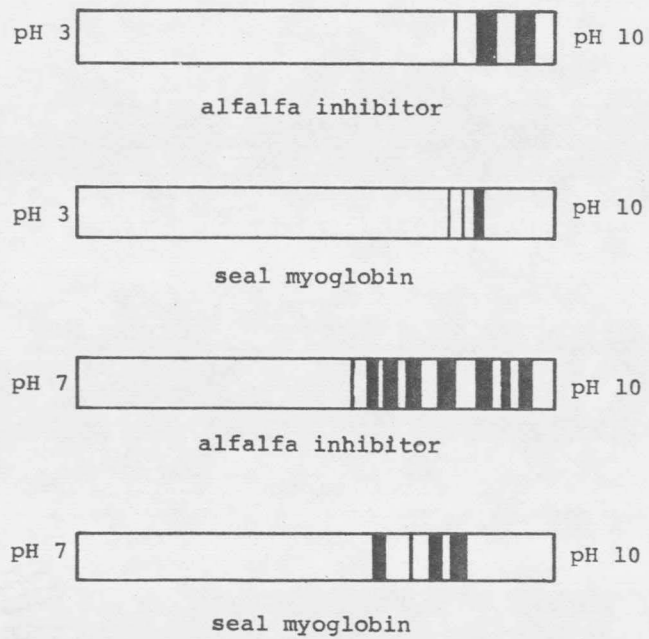


Figure 6. Isoelectric focusing of the alfalfa trypsin inhibitor in narrow and wide ampholyte gradients.

Ampholyte binding. Figure 6 also shows the results obtained when an identical sample of the alfalfa inhibitor was electrofocused in a 7-10 pH gradient. The three bands obtained with the 3-10 pH gradient had split into eight distinct inhibitory bands. The presence of artifacts suggesting excessive heterogeneity of the alfalfa inhibitor was investigated by varying the ratio of ampholine to protein and by rerunning discrete bands. Varying the ratio of ampholyte to protein caused marked alterations in the pattern of zones obtained. At higher ratios, an increase in the number of bands was seen with all bands having a slightly higher apparent isoelectric point.

The two major focused protein bands (pH 3-10 gradient) were fixed with 50% trichloroacetic acid. The individual bands were cut out of the gel and briefly washed to remove the trichloroacetic acid. After elution the protein bands were redissolved in water, and refocused in new gels. As is shown in Figure 7, multiple zones again reappeared.

Frater (74), using an acidic protein from wool, and Kaplan (75) have shown that complexes can form between ampholytes and proteins. This complex formation resulted

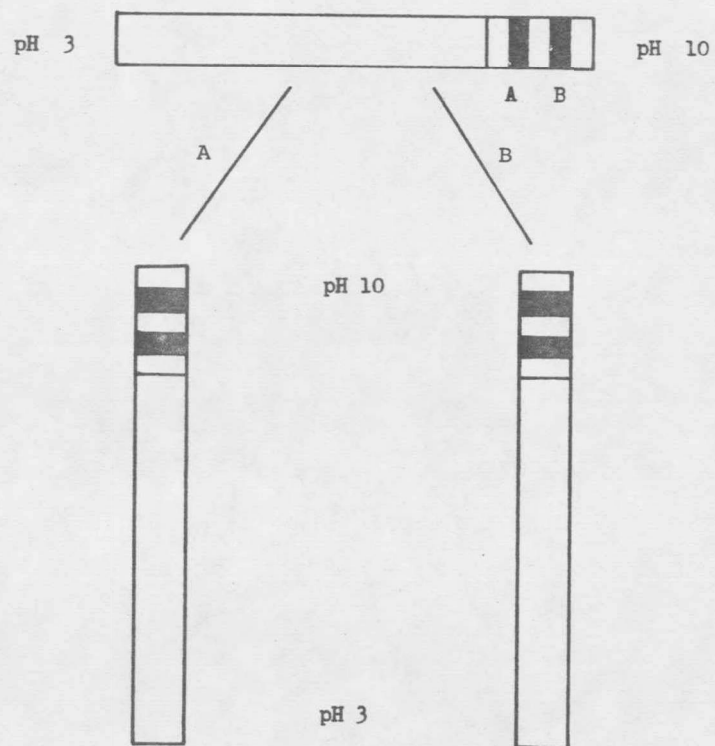


Figure 7. Refocusing of the two major inhibitory bands observed in the pH 3-10 gradient (upper gel Figure 6).

in non-reproducible precipitation patterns which were dependent on concentration. Both researchers also noted that focusing on a wide pH gradient produced several distinct bands which collapsed into a smear upon focusing in a narrow pH gradient.

The artifacts observed might be due to either complex formation, or to the formation of multiple boundaries (76). The positions of the precipitated bands correspond to equilibrium conditions, any alteration of the conditions, such as concentration or point of addition, would result in a different precipitation pattern. The occurrence of artifacts seemed to preclude the use of isoelectric focusing as an indicator of homogeneity of the alfalfa inhibitor system.

Molecular weight determination by gel filtration.

The molecular weight of the inhibitor was determined using the gel filtration method of Anderson (77). Proteins of known molecular weight were chromatographed on Sephadex G-50 and the elution volumes determined. Figure 8 shows the curve relating the elution volume and the log of molecular weight. A sample of alfalfa inhibitor was filtered

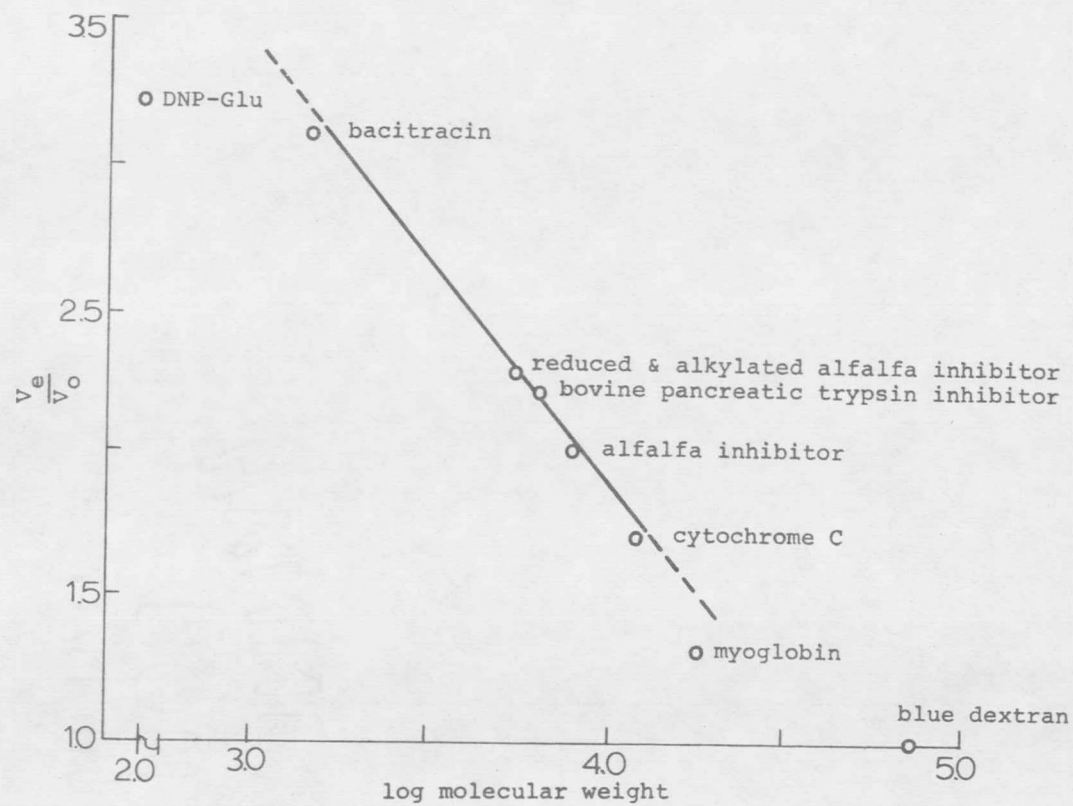


Figure 8. Inhibitor molecular weight determination using Sephadex G-50 gel filtration in 0.05M TRIS·HCl, pH 7.5.

on this column and emerged as a symmetrical peak at a position corresponding to a molecular weight of 7800.

The value for the molecular weight obtained from gel filtration was somewhat larger than the value based on the amino acid composition. The smaller value was based on the occurrence of one residue of histidine per molecule of alfalfa inhibitor. The value 7800 determined from gel filtration was more reliable as it is based on a more direct measurement of molecular weight.

Titration of trypsin with alfalfa inhibitor. The molecular weight of the inhibitor was also determined by titration of trypsin with inhibitor. A TAME assay was used to determine the amount of residual trypsin activity. Figure 9 shows the curve obtained when the trypsin concentration was held constant while varying the inhibitor concentration. Extrapolation of the linear portion of the curve to 100% inhibition allowed calculation of the amount of alfalfa inhibitor bound per mole of trypsin. Assuming that one mole of inhibitor was bound by one mole of trypsin (below), the inhibitor molecular weight was calculated to be 7800 daltons. This value corresponded well with the value obtained from Sephadex chromatography.

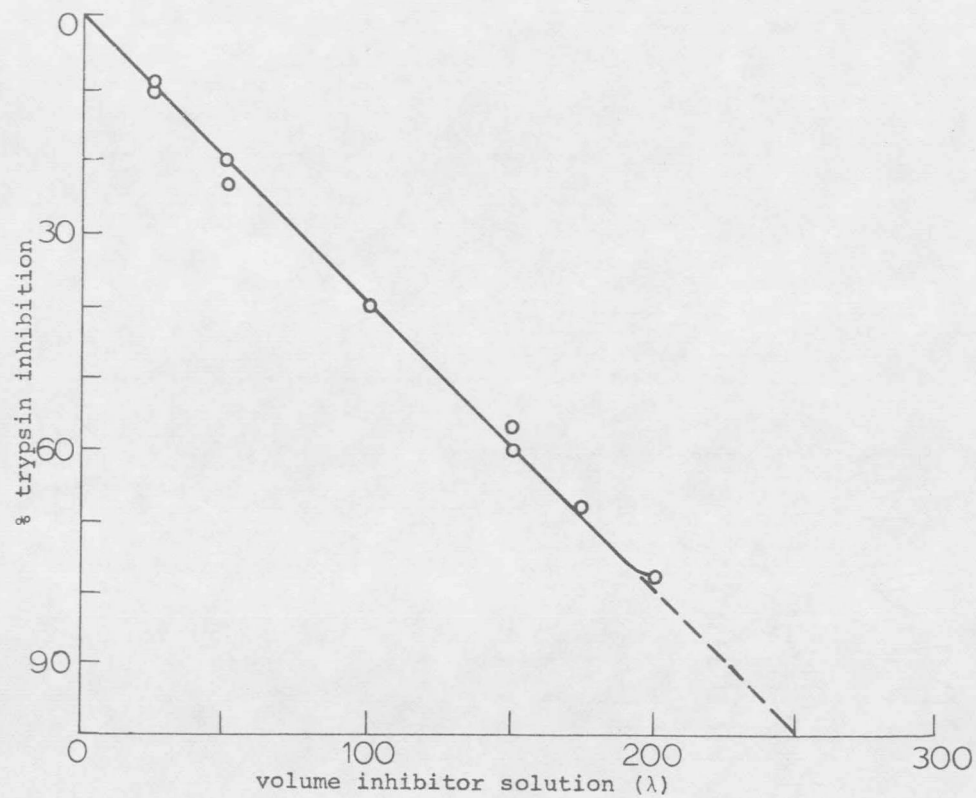


Figure 9. Inhibition of trypsin activity (TAME) by increasing amounts of alfalfa trypsin inhibitor.

Binding characteristics of the complex. The stoichiometry of the trypsin-alfalfa inhibitor complex was determined by use of NPGb, the active site titrant. Figure 10 shows the curve obtained when percent inhibition was plotted vs. the molar ratio of alfalfa inhibitor to trypsin. This experiment was done by holding the trypsin concentration constant while varying the alfalfa inhibitor concentration. Reaction with NPGb gives the amount of trypsin left uncomplexed. Extrapolation of the linear portion of the curve yielded the binding ratio of the trypsin alfalfa inhibitor complex. This extrapolation indicated that one mole of trypsin was bound to 0.96 mole of alfalfa inhibitor. The observed 1:1 binding stoichiometry is characteristic of the low molecular weight proteinase inhibitors isolated from other legumes (2).

Equilibrium dissociation constant. The dissociation constant of the trypsin-alfalfa inhibitor complex was calculated at pH 8.3 from the stoichiometry data present in Figure 10. Calculation of the dissociation constant was done on the extrapolated portion of the curve where the complex was in equilibrium with a measurable

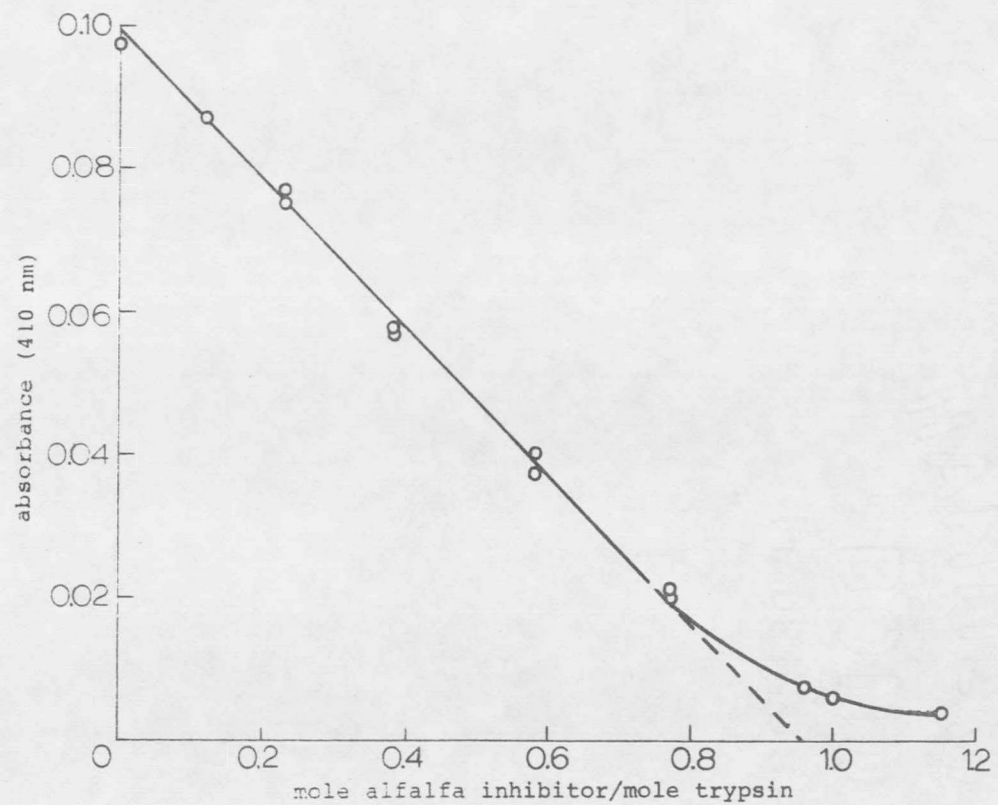
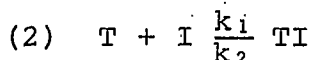


Figure 10. Active trypsin remaining (NPGB) after addition of increasing amounts of alfalfa trypsin inhibitor.

amount of free inhibitor. The calculation described below was done at the point on the curve corresponding to a mole ratio of 0.95 of alfalfa inhibitor to active trypsin.



$$(3) \quad K_{\text{diss}} = \frac{k_2}{k_1} = \frac{[T][I]}{[TI]} \quad \begin{array}{l} [TI] = \text{conc. complex} \\ [T] = \text{conc. free active trypsin} \\ [I] = \text{conc. free inhibitor} \end{array}$$

The initial concentration of the active trypsin ($5.9 \times 10^{-6} \text{M}$) (NPGb) and the alfalfa inhibitor ($5.6 \times 10^{-6} \text{M}$) (weight) was known. The absorbance at this point corresponded to the number of trypsin active sites not in complexation with alfalfa inhibitor. Using an E_{410}^M for p-nitrophenol of 16,370, the amount of free trypsin was calculated to be $4.9 \times 10^{-7} \text{M}$.

Subtraction of the amount of free trypsin from the amount present initially gave the amount of trypsin-alfalfa inhibitor complex present.

initial concentration trypsin	$5.9 \times 10^{-6} \text{M}$
<u>free trypsin after complex formed</u>	<u>$4.9 \times 10^{-7} \text{M}$</u>
concentration of complex [TI] present	$5.4 \times 10^{-6} \text{M}$

Subtraction of the amount of complex present from the amount of alfalfa inhibitor initially present gave the amount of free inhibitor present.

initial concentration alfalfa inhibitor	$5.6 \times 10^{-6} \text{M}$
<u>amount complex present</u>	<u>$5.4 \times 10^{-6} \text{M}$</u>
amt. free alfalfa inhibitor present	$2.0 \times 10^{-7} \text{M}$

The dissociation constant was calculated from equation 3 directly using the concentration values determined above. The calculated value of the dissociation constant was $1.8 \times 10^{-8} \text{M}$ at pH 8.3.

The data presented in Figure 9 was replotted in Figure 11 so that the dissociation constant could be calculated from the data. The TAME assay is a measure of residual enzymatic activity at pH 8.0 in presence of a synthetic substrate. The dissociation constant, using the TAME data, was calculated at a point on the extrapolated portion of the curve corresponding to 80% inhibition.

The concentration of active trypsin molecules before addition of alfalfa inhibitor was determined using NPGB. At the point corresponding to 80% inhibition, the alfalfa inhibitor concentration was $5.1 \times 10^{-7} \text{M}$ while the trypsin concentration was held constant at $5.7 \times 10^{-7} \text{M}$.

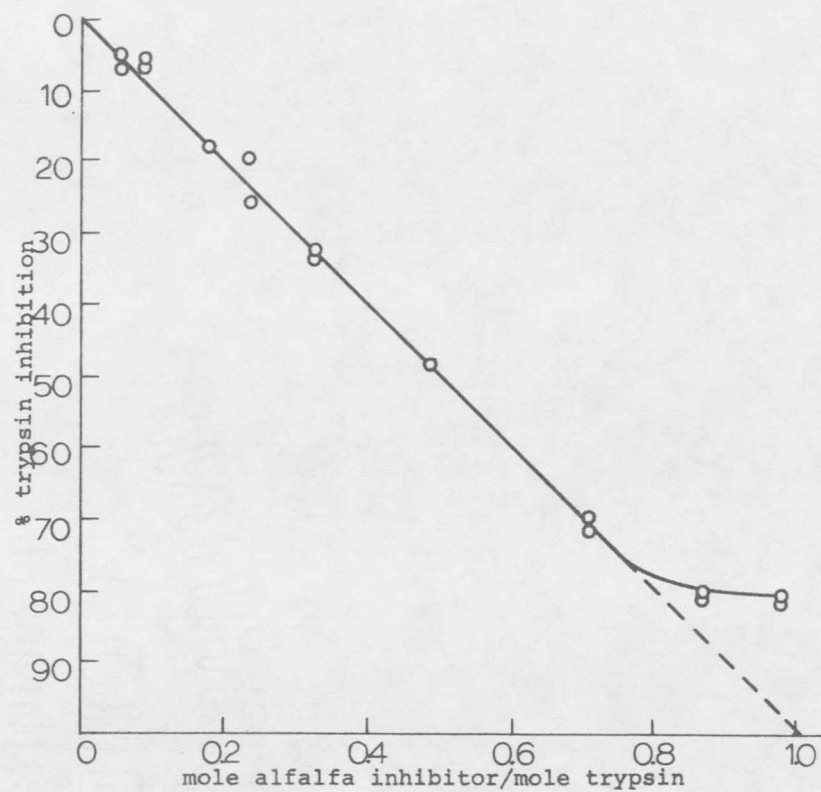


Figure 11. Trypsin inhibition (TAME) in presence of increasing amount of alfalfa inhibitor.

initial concentration trypsin	5.7	$\times 10^{-7}M$
x. % inhibition	.80	
<hr/>		
amt. complex present	4.5	$\times 10^{-7}M$

Subtraction from the initial concentration of both trypsin and alfalfa inhibitor gave the amount of free trypsin or alfalfa inhibitor present.

	<u>trypsin</u>	<u>alfalfa inhibitor</u>
initial concentration	$5.7 \times 10^{-7}M$	$5.1 \times 10^{-7}M$
<u>amount complex</u>	$4.5 \times 10^{-7}M$	$4.5 \times 10^{-7}M$
amt. free	$1.2 \times 10^{-7}M$	$6.0 \times 10^{-8}M$

The dissociation constant was calculated (Equation 3) using the concentration values calculated above to be $1.6 \times 10^{-8}M$ at pH 8.0.

The two equilibrium dissociation constants that were calculated for the trypsin-alfalfa inhibitor complex agreed well with each other. Calculation of dissociation constants from these inhibition curves was valid. Both were calculated by measuring the extent of deviation from stoichiometric inhibition in the region of the equivalence point of a titration. Inhibitor titrations of enzyme are suitable for kinetic calculations if the extent of inhibition at the equivalence point is greater than 70% (78).

Both curves meet this requirement. In addition, the calculations of the dissociation constants are based on the concentration of active trypsin as determined by NPGB assay. The values of the calculated dissociation constant are of the same order of magnitude as those obtained for trypsin inhibitors from soybean (79) and lima bean (80). The affinity for complex formation by trypsin and the alfalfa inhibitor is of intermediate strength when compared to other trypsin-trypsin inhibitor systems.

Rate constants of the trypsin alfalfa inhibitor system. An attempt was made to determine the rate constants for association (k_1) and dissociation (k_2) of the trypsin alfalfa inhibitor system. Satisfactory rate constants describing the binding of trypsin and alfalfa trypsin inhibitor were not obtained due to the reasons described below.

Determination of the dissociation rate constant (k_2) was approached using the NPGB method of Zahnley and Davis (60a). A sample of trypsin was incubated with excess inhibitor and NPGB in sodium veronal buffer at pH 8.3. The apparent dissociation of the complex was followed by monitoring the slow production of p-nitrophenolate ion at

410 nm. The method, as described by Zahnley, appears to be valid only for rapidly dissociating inhibitor system ($k_2 > 10^{-5} \text{sec}^{-1}$). In the case of slowly dissociating systems, the nonspecific esterolysis of NPGB by trypsin and deacylation of guanidinobenzoyltrypsin (and subsequent reaction with more NPGB) resulted in production of p-nitrophenolate in excess of that expected from dissociation of the complex. An experiment containing only trypsin produced more color than did dissociation of a comparable amount of trypsin alfalfa inhibitor complex. This suggested that the rate constant for dissociation (k_2) must be on the order of that for nonspecific esterolysis (10^{-6}sec^{-1}). The method may be valid where the dissociation rate constant to be measured is at least two orders of magnitude faster than the nonspecific esterolysis. Use of nonactive trypsin (DIP-trypsin) in the reference beam would balance out the nonspecific esterolysis allowing the dissociation rate constant (k_2) to be measured. This may lack validity as there would be only a small amount of absorbance due to k_2 in a large background.

The association rate constant was approached using NPGB. This method might be expected to be immune from the

above criticism since the free trypsin remaining is measured via the rapid "burst" method. Trypsin and inhibitor samples were incubated with addition, at various times, of NPGB. The NPGB reacts with all unbound trypsin present at the time it was added. In less than 10 seconds, 80% of the initially free trypsin had complexed with alfalfa inhibitor at 25°C. A lesser concentration of both trypsin and alfalfa inhibitor was used at 10°C in an attempt to slow the reaction to a measurable rate. This was unsuccessful since the concentration of trypsin required (3×10^{-6} M) to give a measurable burst (.05 absorbance units) resulted in an association rate too fast to measure with available instrumentation.

Much lower concentrations (10^{-10} M) of the trypsin could be used in a pH stat to measure residual enzymatic activity of trypsin with TAME. Figure 12 shows the curve that was obtained when trypsin and alfalfa inhibitors were incubated at 4°C and aliquots of the incubation mixture were assayed at time intervals after mixing (81). There was an apparent overshoot of complex formation with subsequent dissociation to an equilibrium value. Laskowski (2) has noted overshoot of complex formation with trypsin and

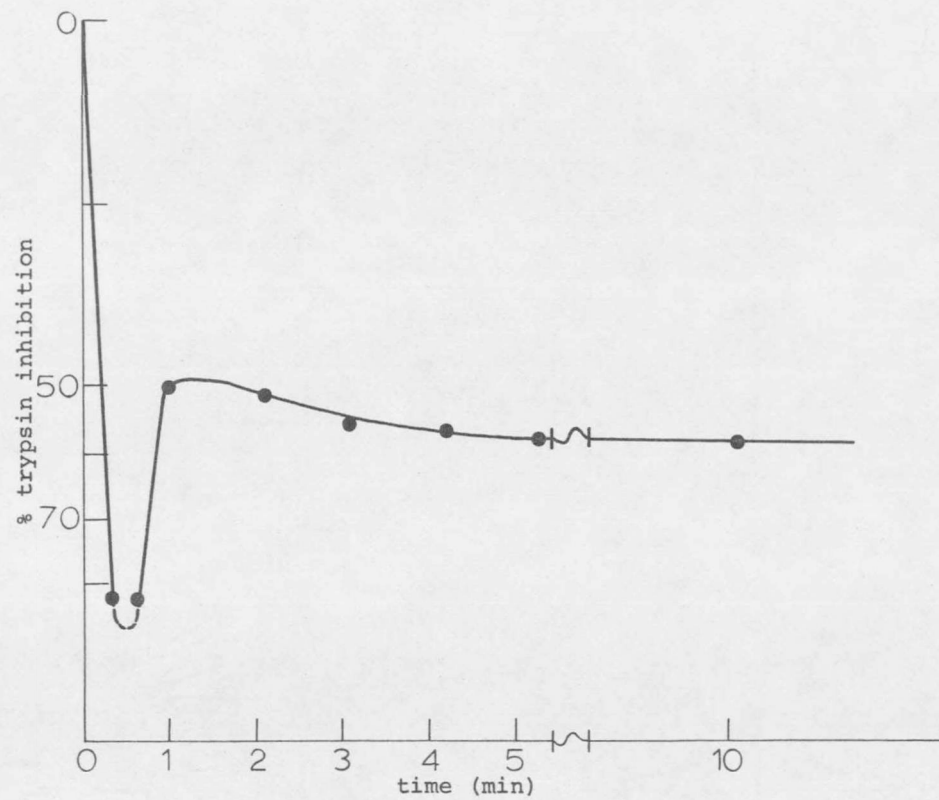


Figure 12. Residual trypsin activity (TAME) after addition of a 1.5 molar excess of alfalfa inhibitor.

soybean trypsin inhibitor using a similar potentiometric assay. He suggested that at least two different reactions followed mixing: the first was the complex association and the second was due to a modification of one or more of the reagents. Therefore, either the trypsin, inhibitor or both present after complex dissociation might not necessarily be the same as the original molecules which entered into the complex. Trypsin (pH 3.75) cleaved the native inhibitor molecule forming a modified inhibitor molecule which does not bind as tightly to trypsin as does the native inhibitor.

Most likely a variation of this mechanism occurs with the alfalfa inhibitor system at neutral pH. Instead of proposing a tryptic cleavage of native inhibitor which would be unlikely at neutral pH, the observed heterogeneity of the alfalfa inhibitor system seems to offer a plausible explanation. Assume that the alfalfa inhibitor system was made up of native inhibitor and modified inhibitor. The native inhibitor would have more affinity for trypsin than would the modified inhibitor (2). When this mixture was added to trypsin, most of the trypsin molecules would complex with native inhibitor causing an overshoot of complex.

As the equilibrium between the trypsin native inhibitor and trypsin modified inhibitor complexes stabilized, more unbound trypsin would be present due to the lesser affinity of the modified inhibitor for trypsin. This equilibrium value would remain constant yielding a straight line such as shown in Figure 12.

As the straight line in Figure 12 corresponded to an equilibrium value, it should then be possible to calculate an equilibrium dissociation constant. The amount of trypsin present initially in the incubation mixture was $2.3 \times 10^{-6} \text{ M}$, while that of inhibitor was $3.5 \times 10^{-6} \text{ M}$. At equilibrium, 35% of the trypsin initially present was still unbound ($0.8 \times 10^{-6} \text{ M}$). Using equation 3, the equilibrium dissociation constant was found to be $6.6 \times 10^{-7} \text{ M}$. This value agrees reasonably well with those calculated above.

The above discussion suggests that the equilibrium dissociation constants obtained for the alfalfa system may well be a hybrid constant describing two or more inhibitor forms. If this is true, then values for the rates of association and dissociation would necessarily reflect a composite of several constants. At the present time a

single homogenous species of alfalfa trypsin inhibitor is unavailable for calculation of association and dissociation rate constants.

Competitive inhibition. If the reaction of alfalfa inhibitor with trypsin is competitive, alfalfa inhibitor and NPGB would be expected to simultaneously compete for the available active sites. A small volume (100 λ) of alfalfa inhibitor (6.3×10^{-6} M) and 10 λ of NPGB (1×10^{-4} M) were mixed. At time zero, 30 λ of active trypsin (5.7×10^{-6} M) was added. The p-nitrophenol burst was about 13% less than what was expected. This indicated that 13% of the active trypsin molecules are inhibited by the alfalfa inhibitor before they can react with NPGB. These results indicated that trypsin inhibition by the alfalfa inhibitor was competitive. Figure 12 shows that the trypsin-alfalfa inhibitor complex undergoes dissociation to reach equilibrium. This supports the reversibility of the trypsin-alfalfa inhibitor complex. The reports on non-competitive inhibition of trypsin by the alfalfa inhibitor were based on Lineweaver-Burke plots using casein as substrate. These plots indicated non-competitive

inhibition due to the small dissociation constant for the complex and the relatively large K_m characteristics of casein and trypsin.

Specificity. The specificity of the alfalfa inhibitor toward various enzymes was examined. After the normal activity of an enzyme against synthetic substrate was determined, an aliquot of enzyme was incubated with an excess amount of alfalfa inhibitor. This aliquot was assayed and compared with the normal activity. The alfalfa inhibitor was determined to be specific for bovine trypsin. It did not inhibit chymotrypsin, elastase, carboxypeptidase A or carboxypeptidase B.

Solubility. The alfalfa inhibitor was soluble in water throughout the pH range 1.5-12. After incubation at various pH's for one hour, no loss of inhibitory activity was detectable by TAME assay. The alfalfa inhibitor was soluble in both 20% trichloroacetic acid and in 90% ethanol. Treatment of disc gels with 50% trichloroacetic acid resulted in initial precipitation of the alfalfa inhibitor which when redissolved in water retained activity.

The precipitated band usually redissolved in the 50% trichloroacetic acid in less than one hour. Inhibitors isolated from bovine pancreas (82) and soybean (83) are soluble in 2.5% trichloroacetic acid and 90% ethanol.

Resistance toward reduction. Reduction of the disulfide bonds of alfalfa inhibitor was attempted using a 25 fold excess (over disulfide) of dithiothreitol. The reduction was attempted in 0.1N sodium borate buffer (pH 8.3) under atmospheric conditions in a 1 ml cuvette. The production of oxidized dithiothreitol was monitored in a spectrophotometer at 310 nm (84). No change in absorbance was noted, indicating that none of the disulfides present in the alfalfa inhibitor were reduced. Iyer and Klee (84) found the use of urea accelerated disulfide reduction of ribonuclease and lysozyme. The alfalfa inhibitor was subjected to disulfide reduction under the same conditions in 8M urea. When no reduction was noted, the concentration of dithiothreitol was increased. No reduction in 8M urea was observed until the dithiothreitol concentration was present in 150 fold excess over disulfide content. The reduced inhibitor had no inhibitory activity toward trypsin. The observed inactivation upon reduction

suggested that structural features dependent on disulfide integrity of the alfalfa inhibitor was necessary for trypsin inhibition.

Heat stability. Figure 13 shows the data obtained when the inhibitor was incubated, at 93°C, in neutral pH buffer. Aliquots of the alfalfa inhibitor solution were allowed to cool before the inhibitory activity against trypsin was determined using a TAME assay. No loss of activity was noted in any of the trials, suggestive of the extreme thermostability of the alfalfa inhibitor. Addition of a 100 fold excess (over disulfide content) of β -mercaptoethanol after two hours incubation of the alfalfa inhibitor at 93°C completely removed the inhibitory activity in less than five minutes. This result supported earlier evidence showing the apparent importance of disulfide bonds for inhibitor activity.

Pepsin hydrolysis. Figure 14 shows the results of incubation at 37°C of a solution of alfalfa inhibitor (0.5 mg) with a catalytic amount of pepsin (0.01 mg). The enzymatic activity of pepsin against hemoglobin was determined and was included in Figure 15. Hydrolysis of

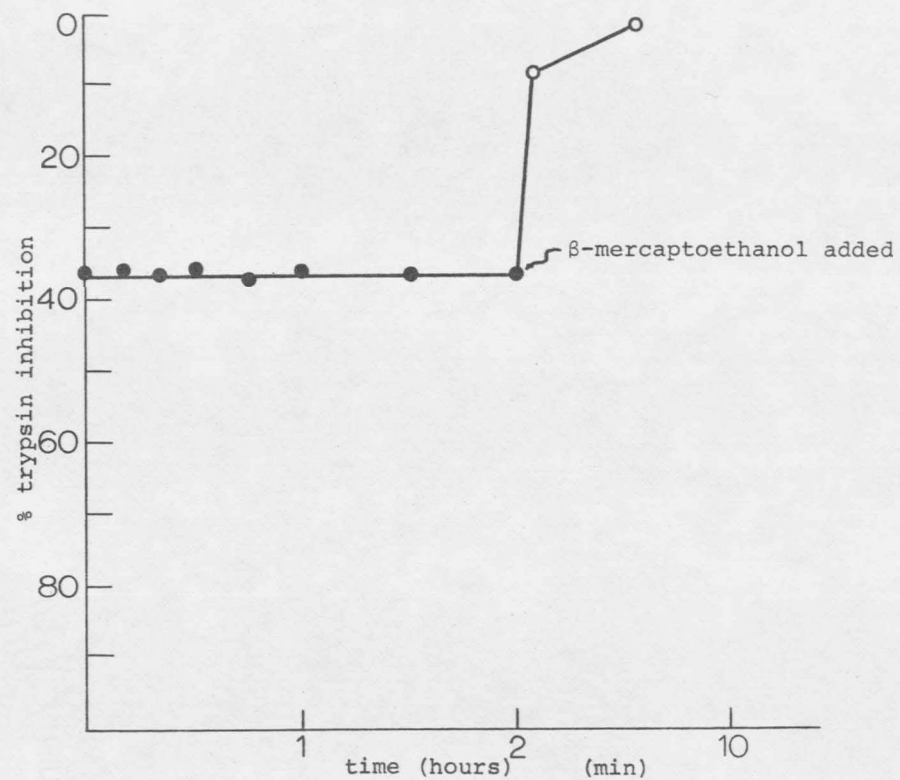


Figure 13. Inhibitory activity of alfalfa trypsin inhibitor after incubation in 0.01M TRIS·HCl pH 7.5 at 93°C. After two hours, β -mercaptoethanol was added to the incubation mixture.

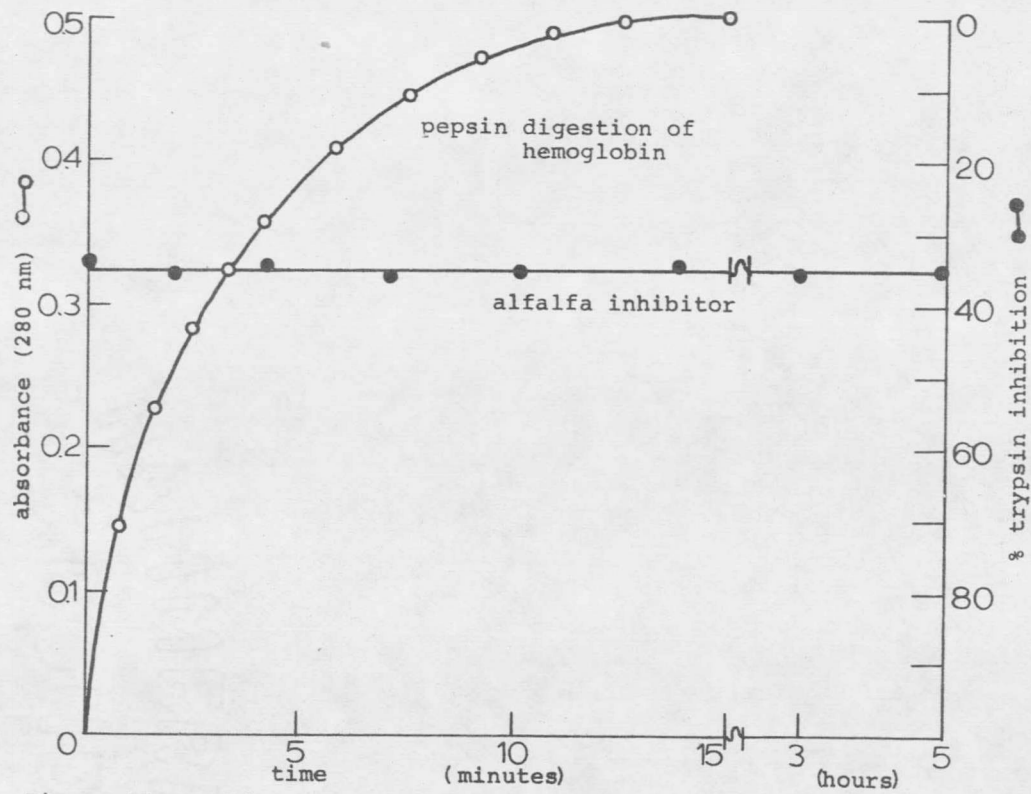


Figure 14. Inhibitory activity of the alfalfa trypsin inhibitor after incubation with pepsin at pH 1.8. The open circles and the left vertical axis represent peptic digestion of hemoglobin under identical conditions.

hemoglobin with pepsin was complete in about 15 minutes. Aliquots of the pepsin alfalfa inhibitor solution were taken every half hour until five hours had elapsed. The alfalfa inhibitor possessed as much inhibitory activity against trypsin at the end of five hours as it had at the beginning as determined by TAME assay. Hence, under the conditions used above, pepsin did not cleave any bonds in the alfalfa inhibitor which are essential for inhibition of trypsin.

The effect of pepsin on the trypsin inhibitory activity of the alfalfa inhibitor is of major nutritional importance. The fact that pepsin does not remove the inhibitory activity suggests that monogastric animal could not utilize the amino acids of the inhibitor since the inhibitors amino acids could not be absorbed without initial degradation. The undigested inhibitor could also be expected to complex with endogenous trypsin and effectively decrease the total enzymatic activity of the gut. As the inhibited trypsin molecule would likely not undergo autolysis, part or all of its amino acids would be lost to the organism. This loss of essential amino acids, notably cysteine, could explain the growth depression noted in

chicks fed raw alfalfa meal (42). This effect has also been suggested as the likely explanation for the growth depression noted in chicks fed raw soybean meal (44).

Chymotrypsin hydrolysis. A catalytic amount of chymotrypsin (0.01 mg) was incubated at room temperature with a sample of the alfalfa inhibitor (0.5 mg). At 20 minute intervals, aliquots were removed and trypsin was added. After a five minute incubation, the samples were subjected to TAME assay to determine trypsin inhibitory activity. No loss of trypsin inhibitory activity was detectible after incubation of the alfalfa inhibitor with chymotrypsin for one hour. Thus, under the condition used chymotrypsin does not cleave any bonds necessary for the trypsin inhibitory activity of the alfalfa inhibitor. The alfalfa inhibitor is not digested by any of the three major enzymes of the gut, trypsin, chymotrypsin or pepsin. The amino acids of the alfalfa inhibitor and some or all of the amino acids of trypsin involved in complex with the inhibitor, therefore, can not be utilized by the organism.

Carboxypeptidase A and B hydrolysis. Samples of the alfalfa inhibitor were incubated with catalytic amounts

of either carboxypeptidase A or B. The digest mixtures, after quenching with sodium citrate (pH 2.2) buffer, were analyzed directly on the amino acid analyzer. Analysis showed that no amino acid residues had been cleaved from the inhibitor. Since the carboxypeptidases are carboxy-terminal exopeptidases, this result indicated that the C-terminal residue of the alfalfa inhibitor was not available for reaction under the conditions used. This unavailability may have been due to masking of the residue or to the presence of a slow to react amino acid such as proline, glycine, aspartic acid, glutamic acid or cystine.

The pepsin and chymotrypsin results showed that no peptide bonds essential for inhibitory activity are cleaved upon incubation of the alfalfa inhibitor with pepsin or chymotrypsin. The high stability of the alfalfa inhibitor to various denaturants, enzyme hydrolysis and to chemical attack all gave indications of the compact structure of the inhibitor molecule. A tight molecular structure is consistent with the high disulfide and proline content.

Carbohydrate content. Samples of the alfalfa inhibitor were subjected to the qualitative test of Dubois (51)

for the presence of sugars and amino sugars. Figure 15 shows a standard curve determined with solutions of known sugar concentration. No change in absorbance was noted when alfalfa inhibitor solutions of varying concentration were subjected to the test indicating that no carbohydrate was present in the alfalfa inhibitor preparation. If only one hexose had been present per mole of alfalfa inhibitor, a change of absorbance corresponding to 23 μ g of glucose would have been expected. The trypsin inhibition observed in alfalfa seeds is therefore due to a protein which does not contain carbohydrate. Mitchell (49) had reported that the trypsin inhibitor present in alfalfa was a glycoprotein. His inhibitor preparations differed in that they were extracts of the vegetative portions of the alfalfa plant. He used a crude preparation which contained some amber colored material which could have erroneously indicated carbohydrate content. At this time, no plant proteinase inhibitors containing carbohydrate have been reported although many animal proteinase inhibitors do contain carbohydrate (2).

Dansylation. A small amount of alfalfa inhibitor was subjected to the dansylation procedure to determine

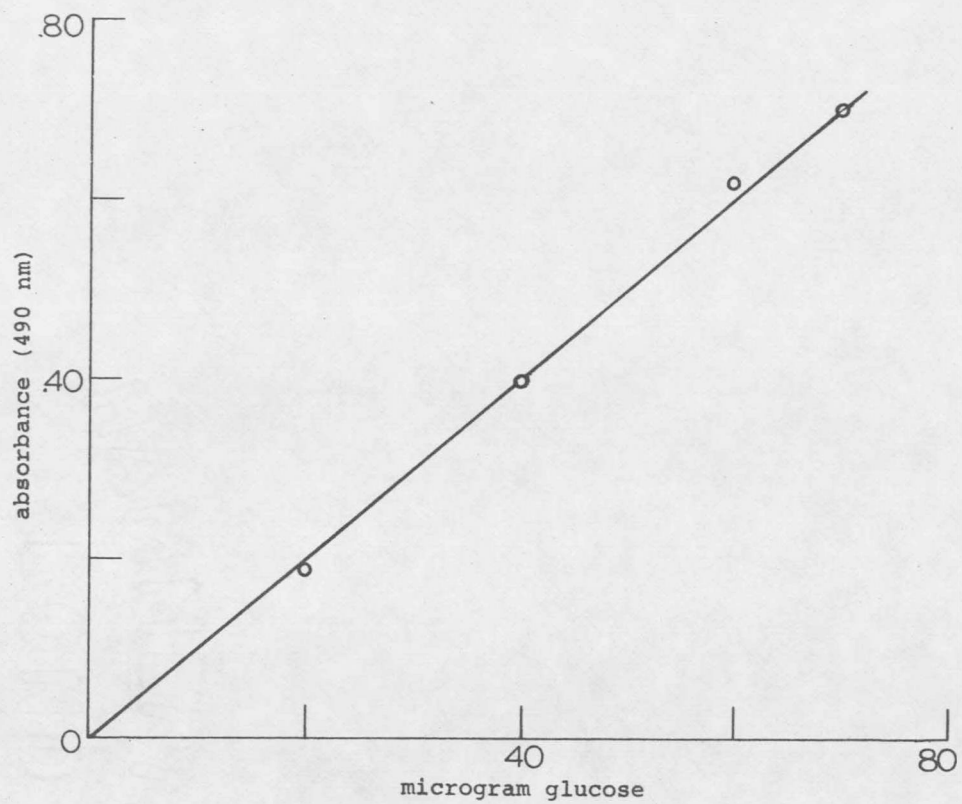


Figure 15. Standard curve for sugar content using the qualitative test of Dubois (51).

the amino terminal residue. After dansylation, thin layer chromatography revealed two spots which corresponded to dansyl-hydroxide and dansyl-amide. It appeared that no detectible reaction of the DNS-Cl had occurred with the alfalfa inhibitor. Dansylation occurred normally when known amino acid samples were treated in a similar manner with DNS-Cl. Thus, it appeared that the amino terminal residue of the alfalfa inhibitor was not available for reaction with DNS-Cl under the conditions used.

Carbamylation. Table 4 shows the results obtained when 1.6 mg of alfalfa inhibitor was reacted with potassium cyanate according to the method of Stark (64). The yields shown in the table were calculated from the initial amount of inhibitor protein and were adjusted for losses inherent to the method. The amount of inhibitor reacted was 0.18 μ M of protein. Significant amounts of four different amino acids were recovered indicating the presence of four free α -amino groups. The presence of glycine may be artifactual as serine and other amino acids yield glycine upon degradation. At least some of the yield observed for glycine is due to degradation. Serine was obtained in

greatest yield with alanine and leucine having about equal yields. This suggested heterogeneity of the alfalfa inhibitor sample. The data could suggest four different inhibitor molecules or inhibitor molecules that had been modified by trypsin. The trypsin susceptible bond in trypsin inhibitors is composed of either an arg-x or lys-x bond. Glycine, alanine and leucine have all been reported as the amino acid X. This suggests that serine and glycine are native amino terminal while alanine and leucine were produced by trypsin cleavage of a susceptible bond. This idea received support when the inhibitor was reduced and alkylated.

Table 4. Amino acids detected after carbamylation (64) of the alfalfa inhibitor

Amino acid	Total residue/mole inhibitor
serine	0.58*
glycine	0.27
alanine	0.35
leucine	0.26

* - yields have been corrected for losses inherent in the method

Reduction and Alkylation

The alfalfa inhibitor was reduced with dithiothreitol (150 fold excess over disulfide content) in 6M guanidine-HCl for 18 hours. After the reduction, carboxamidomethylation of the resultant sulfhydryl groups was achieved by reacting the reduced inhibitor with iodoacetamide for 10 minutes. Mercaptoethanol was added at that time to quench excess iodoacetamide and to preclude over-alkylation. Separation of the reduced inhibitor from the reaction mixture was achieved using gel filtration on Sephadex G-50. Eighty percent of the material subjected to reduction and alkylation was recovered.

The reduced and alkylated inhibitor did not inhibit trypsin which confirmed its functional dependence on the presence of disulfide bonds and presumably native structure.

Amino acid composition. The reduced and alkylated derivative eluted at a volume (tube number 75, Figure 4) corresponding to a lower molecular weight than the native inhibitor upon gel filtration. Reduction and alkylations would interfere with the compact inhibitor structure by

removing the disulfide bridges. The molecule might be expected to appear larger on gel filtration as it would likely have a larger effective radius. The molecular weight of the reduced and alkylated material was determined to be 5500 by gel filtration. Table 5 shows the results of amino acid analysis of the reduced and alkylated inhibitor. Based on one histidine, there were 47 amino acid residues compared to 61 (Table 3) in the native inhibitor. About 11 amino acid residues were missing. These included a lysine, an arginine and three half cystine residues. The presence of three half cystine residues indicated that the missing piece or pieces may have been linked to the major protein chain through at least one disulfide bond. The presence of the basic residues suggests the possibility that the missing amino acids might be part of an arg-x or lys-x sequence located near the amino terminal end of the molecule. The apparent loss of amino acids upon reduction and alkylation has also been noted with a trypsin inhibitor isolated from sainfoin (66).

Carboxyl-terminal determination. The reduced and alkylated inhibitor was incubated with carboxypeptidase B.

Table 5. Amino acid composition of reduced and alkylated alfalfa inhibitor*

Amino acid		Amino acid	
Lysine	2.04	Glycine	1.20
Histidine	1.00	Alanine	1.96
Arginine	2.70	Carboxymethyl-cystine	10.66
Aspartic acid	4.09	Valine	0.13
Threonine	6.98	Methionine	0
Serine	3.27	Isoleucine	3.04
Glutamic acid	2.59	Leucine	0.95
Proline	4.40	Tyrosine	0.73
		Phenylalanine	<u>1.53</u>
			49
Approximate MW		5390	
Total Recovery		90%	

* - based on one histidine

for three hours. Amino acid analysis of the digest mixture revealed that 0.78 residues of arginine were present per mole of reduced and alkylated inhibitor. The carboxyl-terminal amino acid residue of the reduced and alkylated material was arginine. In view of the high yield of arginine obtained, it would appear that no other carboxyl-terminal residues are present. The arginine observed might be the native carboxyl-terminal residue which was unavailable before reduction and alkylation or the product of trypsin cleavage of an arg-x bond. As the amino terminal data suggested heterogeneity of inhibitor molecules, the presence of a single residue as the carboxyl-terminal residue for a number of molecules appears unlikely. The second possibility appeared much more likely after determination of the amino terminal residues of the reduced and alkylated inhibitor.

Dansylation. The reduced and alkylated inhibitor was subjected to dansylation with DNS-Cl. Development on thin layer chromatographic plates yielded three spots. Two of the spots corresponded to dansyl-hydroxide and dansyl-amide. The third spot, reddish-yellow in color,

could not be correlated with any known amino acid. It appeared that reduction and alkylation had no effect on the availability of the α -amino groups to dansyl chloride.

Carbamylation. Table 6 shows the amino terminal residues, with their apparent yields, of the reduced and alkylated material. Of the four residues (serine, glycine, alanine and leucine) observed with the native inhibitor, only serine and glycine were still present in the reduced and alkylated inhibitor. The presence of the same two amino terminal residues in the native and the reduced and alkylated inhibitors indicates the possible presence of two types of inhibitor molecules. The loss of alanine and leucine from the reduced and alkylated inhibitor, with the concurrent appearance of arginine as carboxyl-terminal, suggest the possibility of two modified inhibitors of the arg-x type. Further experimentation would be required to determine the true state of affairs.

Heterogeneity of the Alfalfa Trypsin Inhibitors

Carboxymethyl cellulose chromatography. Ion exchange chromatography of the major alfalfa inhibitor peak from gel filtration (Figure 4) is shown in Figure 16.

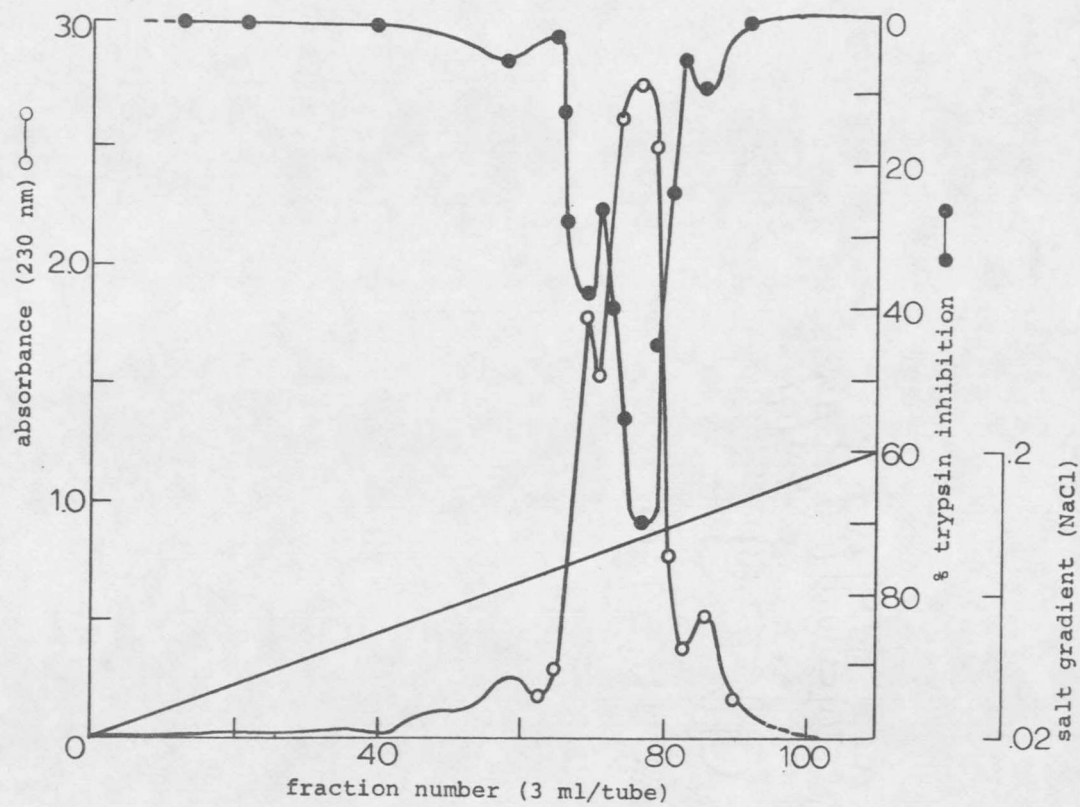


Figure 16. Ion exchange chromatography of the alfalfa trypsin inhibitor on carboxymethyl cellulose in pH 5.0 sodium acetate buffer.

The separation was done at pH 5.0 using a linear salt gradient .02 - .2N in Na^+ . The alfalfa inhibitor split into a number of peaks, one of which predominated. The large peak was rechromatographed under the same conditions and as Figure 17 shows, emerged as a single peak, somewhat unsymmetrical on its leading edge. This peak was rechromatographed, as shown in Figure 18, using a narrow salt gradient (0.05 - 0.15 in Na^+). Two peaks, B_1 and B_2 , of unequal size were obtained. The amino acid composition of B_1 and B_2 was determined. Table 7 shows the composition of B_1 and B_2 as well as peak A from Figure 16. The amino acid analysis data clearly shows that the isolated peaks differ both in composition and in size. Both peaks contained inhibitory activity toward trypsin. This suggested that at least two major inhibitors may be present in alfalfa.

Table 6. Amino acids detected after carbamylation of reduced and alkylated alfalfa inhibitor

<u>Amino terminal^Δ</u>	
<u>Amino acid</u>	<u>Total residue</u>
serine	0.69
glycine	0.20

Δ - determined by cyanate method

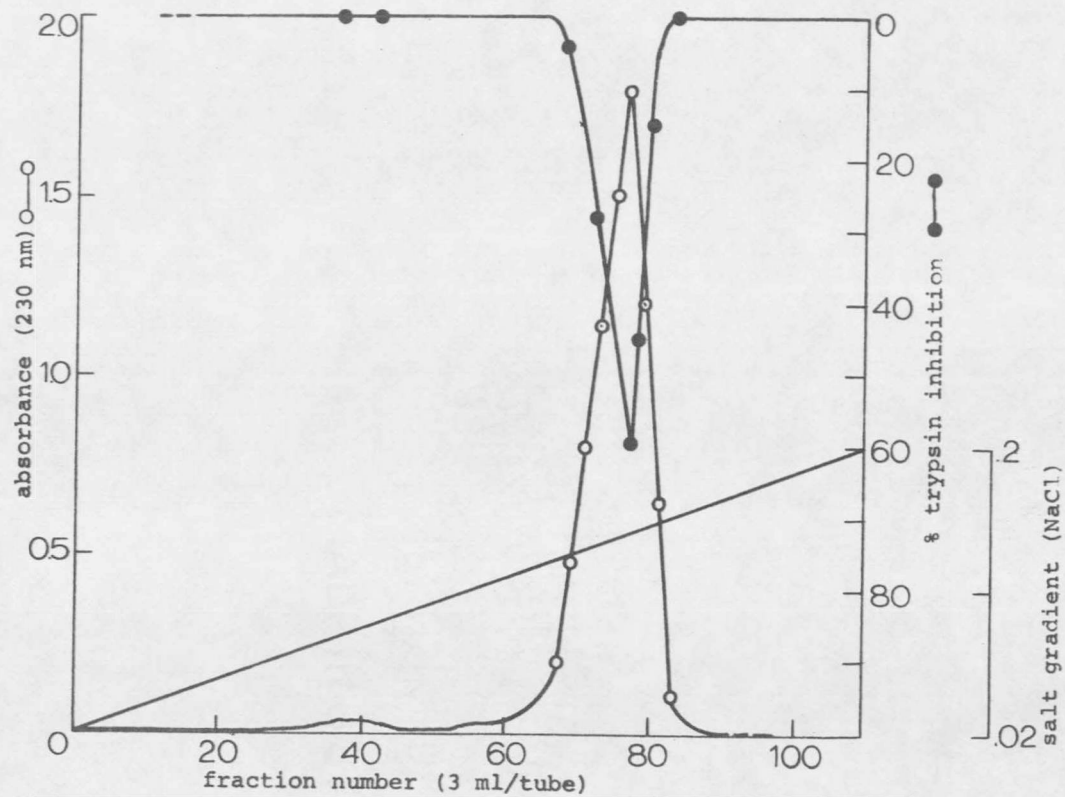


Figure 17. Rechromatography of the major inhibitory peak (Figure 16) on carboxymethyl cellulose in pH 5.0 sodium acetate buffer.

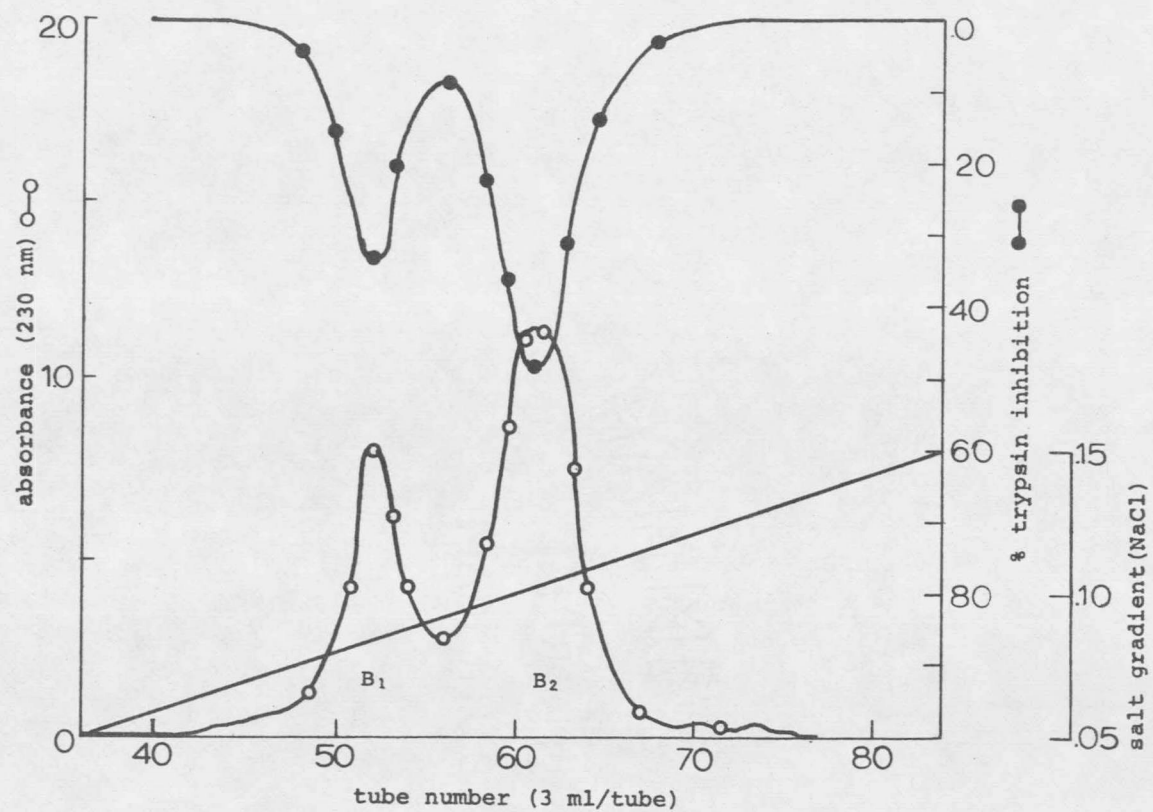


Figure 18. Rechromatography of the inhibitory peak from Figure 17 on carboxymethyl cellulose in pH 5.0 sodium acetate buffer. The salt gradient was broader than that in Figure 17.

Table 7. Amino acid composition of inhibitory peaks isolated from alfalfa using carboxymethyl cellulose*

Amino acid	Peak A	Peak B ₁	Peak B ₂
Lysine	2.46	2.06	2.16
Histidine	0.99	1.08	1.04
Arginine	3.63	2.26	3.70
Aspartic acid	5.24	4.10	5.16
Threonine	7.69	6.81	8.32
Serine	3.59	3.01	4.06
Glutamic acid	3.28	2.78	3.29
Proline	6.27	4.45	6.13
Glycine	1.35	1.05	1.20
Alanine	2.87	1.92	2.06
Cystine	11.69	10.18	13.13
Valine	0.03	0.33	0.28
Methionine	0	0	0
Isoleucine	3.98	2.96	4.09
Leucine	1.04	1.00	1.00
Tyrosine	0.84	0.61	0.50
Phenylalanine	<u>1.98</u>	<u>1.53</u>	<u>2.02</u>
	57	46	58
Approximate MW	6270	5060	6380
Total Recovery	60%	85%	80%

* - based on one histidine

The possibility of heterogeneity in the alfalfa inhibitor system was supported by both carbamylation and ion exchange chromatography data. The non-integral ratios observed in the amino acid compositions lends support for heterogeneity.

It seems likely that alfalfa, like other plants (10), has more than one protein which inhibits trypsin. This class of proteins appears to be of similar size and composition suggesting the possibility of an ancestral gene which has undergone either gene duplication and/or point mutation to produce the observed variability of inhibitory species (85).

CONCLUSIONS

Ground alfalfa seeds contained significant amounts of a protein trypsin inhibitor and little, if any, chymotrypsin inhibitor. The protein trypsin inhibitor isolated accounted for 0.13% of the total protein of the alfalfa seed. High yield isolation of the trypsin inhibitor was accomplished by selective adsorption to insolubilized trypsin-sepharose with subsequent elution upon lowering the pH of the eluting buffer. The insolubilized trypsin was determined to be capable of acylation by NPGB. It could not, however, effectively undergo deacylation as measured by a TAME assay. The apparent lack of enzymatic activity did not prevent inhibitor binding. It was observed that 1.75 mg of alfalfa inhibitor could be bound per ml of sepharose-trypsin beads.

The isolated trypsin inhibitor was subjected to a number of tests for purity. Both disc gel electrophoresis and gel filtration chromatography indicated the presence of one major inhibitory species. Ion exchange chromatography separated the inhibitory activity into a number of peaks. Carbamylation of the alfalfa inhibitor yielded a number of amino-terminal residues. Most likely, there are

at least two different species of trypsin inhibitors present in alfalfa.

In the course of this work, the unusual binding between the alfalfa inhibitor and the ampholytes used in isoelectric focusing was investigated. Upon isoelectric focusing of the alfalfa inhibitor ampholyte system, equilibrium band position was noted that was concentration dependent. When any one band was refocused, it would reproduce the entire pattern of bands. This observed binding is the first reported occurrence in this class of protein.

The alfalfa inhibitor was soluble in the pH range 1.5-12, in 20% trichloroacetic acid and in 90% ethanol. High temperature had little, if any, effect on inhibitory activity of the alfalfa inhibitor. In view of the above, it is easy to understand why reduction of the alfalfa inhibitor must be done under rigorous conditions. The alfalfa inhibitor appeared to be stable to most denaturing solutions and has remarkable stability against hydrolysis by enzymes such as pepsin, chymotrypsin and carboxypeptidase A or B.

When one examined the amino acid composition of the alfalfa inhibitor, the unusual stability became easier to

understand. The alfalfa inhibitor contained 58 amino acid residues with an approximate molecular weight of 6380. Of these, 23 percent was present as half cystine (14 residues/molecule) and 10 percent as proline. This suggested that the inhibitor had a very tight compact structure. The inhibitor also lacked methionine, tryptophan and most likely valine.

When the molecular weight was calculated by gel filtration or trypsin titration, a value of 7800 daltons was obtained. The value of 6380 determined from amino acid compositions was dependent on the assumption that there was only one histidine present per molecule of alfalfa inhibitor. If the alfalfa system contained a number of different species with varying amounts of histidine, the value for the molecular weight might be expected to be low.

After the molecular weight was determined, the binding stoichiometry of complex formation was determined to be one mole of trypsin per one mole of alfalfa inhibitor. The equilibrium dissociation constant of the trypsin alfalfa inhibitor complex was calculated at both pH 8.3 and at pH 8.0. Both a direct measurement of unbound

trypsin (NPGB) and a measure of residual enzymatic activity yielded a value of $1.6-1.8 \times 10^{-8} \text{ M}$ for the equilibrium dissociation constant. An additional equilibrium dissociation constant was calculated by allowing trypsin and excess inhibitor to reach an equilibrium. The calculated value was $6.6 \times 10^{-7} \text{ M}$ which agrees well with the other values determined.

A number of attempts were made to calculate rate constants of association and dissociations of complex, but were unsuccessful. The NPGB system could not be used to determine either the dissociation rate constant or the association rate constant. The dissociation rate constant (k_2) of the complex was of the same order of magnitude as the rate constant for nonspecific esterolysis of NPGB by trypsin while the association reaction proceeded so rapidly that it could not be measured on available instrumentation. When a potentiometric assay (TAME) was used in an attempt to determine the association rate constant (k_1) an interesting phenomenon occurred. Initially, about 80% of the trypsin was inhibited with a slow liberation (4 min) of trypsin until a steady state value of 65% inhibition was reached. This indicated the presence in the alfalfa

system of two or more inhibitors which have different affinities for trypsin. Thus, the calculated equilibrium dissociation constant was most likely a hybrid with contributions from several inhibitor species.

The alfalfa inhibitor was subjected to enzymatic hydrolysis to determine the carboxy-terminal amino acid. This was unsuccessful indicating that this residue must not be available for enzymatic attack. Carbamylation yielded four α -amino groups which presumably corresponded to amino-terminal amino acids. Most likely the glycine obtained was due to degradation of the actual amino-terminal amino acids. Of the three possible amino-terminal amino acids, serine was present in highest concentration, with alanine and leucine present in lesser concentrations.

Reduction and alkylation of the alfalfa inhibitor was accompanied by total destruction of inhibitory activity. This procedure resulted in a material which had a molecular weight of 5390. A total of eleven amino acids were missing, including a lysine, an arginine and three half cystine residues. The presence of the odd number of half cystine residues indicated that the missing amino acids were likely joined to the major protein chain through

at least one disulfide bond. Arginine was determined to be the carboxy-terminal amino acid in the reduced and alkylated inhibitor. The amino-terminal amino acid was determined by carbamylation to be serine. The lack of alanine and leucine as amino-terminal amino acids in the reduced and alkylated inhibitor suggested that they were likely amino-terminal to the missing fragment. Serine was most likely the amino-terminal amino acid for all of the native inhibitor molecule present. This would place the active site of the alfalfa inhibitor in the carboxy-terminal portion of the molecule approximately fourteen residues internal. The occurrence of arginine as the carboxy-terminal amino acid in the reduced and alkylated inhibitor lent credence to this theory since trypsin inhibitory sites are of either an arginine-x or lysine-x type. Both alanine and leucine have been reported in the x-position in other trypsin inhibitors. Further experimentation would be necessary to determine the actual structure of the alfalfa inhibitor.

The nutritional aspects of this system deserved some comment. As the inhibitor was stable to both peptic and chymotryptic hydrolysis and extremes in pH and

temperature, most likely it can not be degraded in the gut of monogastric animals. The small loss of protein content (0.13%) due to the alfalfa inhibitor would have little effect on the animal. Other effects, however, might occur. Raw soybean meal (trypsin inhibitor = 6.0% total protein) is known to cause both growth depression and pancreatic hypertrophy. As the alfalfa inhibitor only contains 0.13% of the total protein as trypsin inhibitor, it is not known what effect feeding alfalfa would have on a normal growing animal. It is known, however, that a 5% diet of alfalfa meal causes growth depression in chicks. The total effect of the trypsin inhibitor could only be determined by feeding studies.

Future work to be undertaken on the subject would include isolation of the several inhibitors and characterization of them. Feeding experiments, to determine the effects on monogastric animals, and physiological experiments, to determine the role in the alfalfa seed, will likely be undertaken. Finally, after the effects and function of the alfalfa inhibitor are known, the plant breeder may wish to decrease or enhance the concentration of inhibitor in appropriate crops.

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