Isolation and characterization of trypsin and chymotrypsin inhibitors from barley
by Casey Gwo-perng Tzeng

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Chemistry
Montana State University

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Naturally occurring proteinase inhibitors were isolated and characterized from two varieties of - barley, Waxy Compana and Hiproly. Affinity chromatography columns of sepharose-trypsin and sepharose-chymotrypsin were used to absorb the inhibitors from neutral aqueous extracts of barley meal. Both trypsin and chymotrypsin inhibitors were present at concentrations of .02-.04 g inhibitor per 100 g seed. Multiple inhibitory bands of both inhibitors were observed upon electrophoresis. The proteins are monomeric in solution with molecular weights ranging from 14,000-18,000. No carbohydrate was associated with the inhibitors. Amino acid analysis showed 5-6 disulfide bonds in the trypsin inhibitor and one disulfide bond in the chymo-trypsin inhibitor. Both lacked tryptophan. The chymo-trypsin inhibitors were heat labile whereas both inhibitor types were inactivated by digestive proteinases. It was concluded that the proteinase inhibitors represent no potential physiological or nutritional problems for human and animal consumption of barley products.

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Date Dec. 9. 1974

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ABSTRACT

Naturally occurring proteinase inhibitors were isolated and characterized from two varieties of barley, Waxy Compana and Hiproly. Affinity chromatography columns of sepharose-trypsin and sepharose-chymotrypsin were used to absorb the inhibitors from neutral aqueous extracts of barley meal. Both trypsin and chymotrypsin inhibitors were present at concentrations of .02-.04 g inhibitor per 100 g seed. Multiple inhibitory bands of both inhibitors were observed upon electrophoresis. The proteins are monomeric in solution with molecular weights ranging from 14,000-18,000. No carbohydrate was associated with the inhibitors. Amino acid analysis showed 5-6 disulfide bonds in the trypsin inhibitor and one disulfide bond in the chymotrypsin inhibitor. Both lacked tryptophan. The chymotrypsin inhibitors were heat labile whereas both inhibitor types were inactivated by digestive proteinases. It was concluded that the proteinase inhibitors represent no potential physiological or nutritional problems for human and animal consumption of barley products.

INTRODUCTION

General

It has long been known that some plant proteins can inhibit the action of certain mammalian enzymes. The first member of this group of biologically active proteins to be recognized was trypsin inhibitor from soybeans (1). Since inhibitors have been found in a variety of plant tissues, these proteins are often considered from a nutritional point of view and have been regarded as curiosities but their physiological roles in plants have not been identified. The inhibition spectra of the inhibitors vary considerably. Some are strictly specific, inhibiting only one enzyme, while others are polyvalent and can inhibit several enzymes.

Read and Haas were the first to recognize the presence of an inhibitor of trypsin in plant materials (2). The realization that proteinase inhibitors might be of nutritional significance in plant foodstuffs, particularly in such an important dietary source of protein as legumes, stimulated research for similar factors in other plants. Most of the proteinase inhibitors so far observed have been found in the seeds of various plants but they are not necessarily restricted to this part of the plant. The

observation that inhibitor concentration is relatively high in young growing tissue, but low in older tissue (3), suggests that the inhibitors may play an important role in the regulation of protein metabolism. The ability of potato plant tissue to respond to insect injury by accumulating large quantities of the inhibitors (4) suggests that these inhibitors may serve to make the plant less palatable and perhaps lethal to invading insects. Digestibility of food is known to be an important factor in plant selection by leaf-eating insects (5). The effectiveness of proteinase inhibitors as a deterrent to insects would depend upon their ability to inhibit the proteinases in the insect digestive tract.

The plant proteinase inhibitors are generally small proteins having molecular weights of under 50,000 and more commonly under 20,000 (6). Nearly all plant inhibitors inhibit enzymes of animal or microbial organisms and have either trypsin-like or chymotrypsin-like specificities.

The emerging picture from structural and specificity similarities among plant inhibitors from different sources indicates that the active inhibitor sites may have been conserved over millions of years of evolution and suggests that the inhibitory capacity is important for

survival (7). This, together with recent advances into the physiology of inhibitors in plants, suggests that the inhibitors may have important roles as; a) regulating agents in controlling endogenous proteinases, b) storage proteins, c) protective agents directed against insect or microbial proteinases. An example of an inhibitor effective against an endogenous plant proteinase is the system in barley. The seeds contain three groups of proteolytic inhibitors; an Aspergillus proteinase inhibitor, trypsin inhibitor and inhibitors of endogenous proteinases (8). During germination, the inhibitors of endogenous proteinase are rapidly destroyed while the other two remain unchanged. The decrease in inhibitor content is accompanied by an increase in activity of the plant's proteolytic enzymes (8).

The earlier literature on the chemical and physical properties of the proteinases inhibitors has been reviewed (9). Pharmacological properties of inhibitors and their possible clinical uses have been under investigation (10). Papers dealing with various aspects of proteinase inhibitors continue to appear in ever increasing numbers, but many facets of the subject are still controversial and unexplained. The physiological significance of plant

proteinase inhibitors is only beginning to be investigated.

Definition of Proteinase Inhibitors

Protein proteinase inhibitor is a protein which can associate reversibly with proteolytic enzymes to form complexes in which all of the catalytic functions of the proteinase are competitively inhibited. The inhibitors usually have molecular weights in the range 5,000-60,000, usually less than 20,000. The inhibited proteinases are usually endopeptidases, i.e., peptidyl-peptide hydrolases, although there are reports of protein inhibitors of carboxypeptidases (11). Typically, the proteinase inhibitors have high proline and disulfide content suggestive of their compact structures, and low amounts of tryptophan, histidine, cysteine and methionine. All inhibitors contain an "active site" which confers upon the inhibitor its specificity toward proteolytic enzymes (12). The trypsin specific inhibitors always have either LYS-X or ARG-X (X=Ile, Ala) at the binding site, whereas chymotrypsin specific inhibitors usually have LEU-X (X=Ser) at their active center (13). The mechanism of inhibition has been studied carefully. Finkenstadt and Laskowski, Jr. (14,15)

postulated that the complex is an acyl intermediate between the COOH group of the reactive site residue of inhibitor and the active site serine of the enzyme. This view was, however, subjected to considerable criticism. For example, Ryan and Foster (16) and Feinstein and Feeney (17) found that catalytically inactive enzymes which can not be acylated by substrate, can still bind proteinase inhibitors effectively. The assembly of the three dimensional structure of α-chymotrypsin and inhibitor determined by X-ray crystallography has given a model of the structure of the complex (18,19). The interactions which stabilize the complex are seven hydrogen bonds and the probable formation of a persistent "tetrahedral adduct bond" which links lysine-15 of the inhibitor (the α-carbonyl function) and serine-195 of the active site of α-chymotrypsin (the hydroxyl group).

Nutritional Significance of Proteinase Inhibitors

It has been recognized for a long time that a ration containing raw soybean inhibits growth in rats, chickens and some other monogastric animals. The obvious implication is that the soybean inhibitors of proteolytic enzymes are responsible for this effect. However,

Gertler et al. concluded that factors other than trypsin inhibitor may actually be responsible (20). In addition, many studies on the effect of chicken egg white in humans must be reconsidered in the light of the observation that the principal inhibitor in egg white, ovomucoid, does not inhibit human trypsin, although it inhibits bovine trypsin (21).

Nevertheless, the nutritional studies with laboratory and farm animals indicated a relationship between the presence of the soybean inhibitor and the growth retardation effect. Soybean trypsin inhibitor enhances the formation or release of a humoral pancreozymic-like substance that markedly stimulates external secretion of the rat pancreas (22). When the plasma from rats that were fed soybean trypsin inhibitor was perfused through an isolated rat pancreas, amylase secretion was increased two to three times that of a pancreas perfused with plasma from rats fed the same diet without the trypsin inhibitor. Hyperplasia of some of the pancreatic cells occurs as a result of feeding trypsin inhibitor (23,24). Some investigators believe that insofar as growth retardation is concerned, the effect is primarily a nutritional one and is caused by unavailability of amino acids. It has been suggested that

in the case of navy bean, there is a disproportionately high amount of cystine in the trypsin inhibitor, and that the poor digestibility of the inhibitor leads to a deficiency in cystine (25). Unfortunately, in spite of the extensive research activity and the apparent excellence of some of the investigations, the answer to the nutritional and physiological significance of inhibitors is still not clear.

Distribution of Proteinase Inhibitors

Plant. Trypsin inhibitors are distributed widely in legume seeds (6) and have been investigated extensively because of possible adverse effects on protein digestion when ingested by animals. More recent research has shown that they are present also in other plant tissues such as sweet potato (26), beet (6), alfalfa leaves (27), cereal grains (6) and lettuces (28). Table 1 lists most of the proteinase inhibitors found in various plants (29) and animals (12). In sweet potato, a trypsin inhibitor is found not only in the tuber but also in the leaves (26). It is also noted that in the double bean and field bean, trypsin inhibitors are distributed throughout all parts of

Table 1. Distribution of Proteinase Inhibitors in Plants (29)

Common name	Part of plant
Peanut	seed, skin (38)
Oats	seed (39)
Beet	root (6)
Field bean	all parts (30)
Double bean	all parts (40)
Buckwheat	seed (39)
Soybean	seed (6)
Kentucky coffee bean	seed (41)
Sweet potato	root and leaves (26)
Lettuce	seed (28)
Alfalfa	leaves (27)
Rice	seed (39)
Mung bean	seed and leaves (42)
Lima bean	seed (43)
Navy bean	seed (44)
Garden bean	seed (45)
Rye	seed (46)
Corn	seed (47)
White potato	root and leaves (48)

Distribution of Proteinase Inhibitors in Animals (12)

Sources	Enzymes inhibited
Egg white	Tryp. (49)
Tinamou ovomucoid	Chym. (50)
Turkey ovomucoid	Tryp. Chym. Subtilisin. (51)
Penguin	Tryp. Chym. Subtilisin.
Pancreas	
Bovine tissue	Tryp. Chym. (52)
Bovine juice	Tryp. (53)
Porcine juice	Tryp. (54)
Blood	
Human	Tryp. Chym. (32)
Bovine	Tryp. Chym. (55)
Ovine	Tryp. Chym. (56)
Colostrum	
Bovine	Tryp. (21)
Porcine	Tryp. (57)
Ascaris	Tryp. (58)

the germinating seed and growing plant, but the levels vary depending on the stage of growth (30).

Animals. The first reports on the inhibition of proteolytic enzymes by substance from body tissues date back to the turn of the century, when these materials were referred to as "anti-enzymes". In the human organism, they have been found in urine (31), blood serum (32), sublingual glands (33), semen (34), lymph nodes (31), liver, lung, pancreas, nasal secretion, mucous membrane of the respiratory passage and skin (6). In addition to the mammals, proteinase inhibitors have so far been found in nematodes and birds. The presence of proteolytic inhibitors in intestinal parasites was early observed by Mendel and Blood (35). Proteinase inhibitors have also been detected in microbial organism cultures such as Clostridium botulinum (36) and Aspergillus soyae (37). The emerging picture suggests that protein proteinase inhibitors are ubiquitous throughout the plant and animal worlds.

Function and Roles of Proteinase Inhibitors

The most attractive idea for a general role of naturally occurring protein inhibitors is that they control the action of proteolytic enzymes or esterases in the many

different tissues and fluids in which both the inhibitors and the enzyme occur. For example: a) the control of activation of zymogens or precursors to other biologically active substances: one of the most obvious places where these inhibitors might have a function is in the pancreas where they could regulate activation of the zymogens, trypsinogens and chymotrypsinogens by trypsin (59). Both the zymogens and the inhibitors are present in the pancreas, and the inhibitor may serve as a control to prevent the premature activation of the zymogens, if a small amount of trypsin should be formed. b) at least two inhibitors in blood serum have been demonstrated to inhibit certain of the blood clotting enzymes. The blood clotting system is delicately balanced and an additional control such as an inhibitor of one or more of the clotting enzymes would be a facile way to prevent undesired clotting in the general circulation (60). c) a common feature among various types of inflammation is the accumulation of protein at or near the site of injury. Local conditions frequently favor denaturation, heat aggregation or fibrin formation. These altered proteins must ultimately be digested or removed for the completion of healing (61). The inhibitor can stop the enzymatic action of one of

these enzymes and thereby prevents the inflammatory process. It was demonstrated that when an endotoxin and a trypsin inhibitor were injected intradermally, the inflammatory reaction did not occur.

Plant inhibitors are usually found in the storage organs of plants. There is evidence that they form complexes with proteolytic enzymes or other enzymes. Recently, a change in the concentration of one of the potato inhibitors in the leaflets of young growing potato plant was observed during maturation of the plant (48). There was a direct correlation between the presence of an inhibitor in normally growing young potato leaves and apical rhizome growth. It had been found that the proteinase inhibitors are produced throughout the plant tissues in large quantities in response to insect or mechanical wounding of a single leaf of potato (62). The accumulation of inhibitor is conceivably an important immune response directed against insects or micro-organisms. The response is mediated by a hormone-like factor released from the wound site called potato inhibitor inducing factor (PIIF). Obviously, many more studies are necessary in plant biochemistry and plant physiology before a

complete understanding of the function of these proteins in plants will be achieved.

One apparent application of the inhibitor is its use in the food industry. Protein inhibitors might have an application in controlling proteolytic enzymes in the processing of foods. Aside from the nutritional aspects of enzyme inhibitors, there are several ways that these proteins may be important in food processing. Fruit and vegetable quality might be maintained by storage conditions conducive to a favorably altered balance of inhibitors and degradative enzymes. Varieties with improved storage properties could be developed by selection for high levels of enzyme inhibitors (63).

Future Research of Proteinase Inhibitors

Plant tissues, particularly germinating seeds, leaves, flowers and fruits are valuable systems for studying the roles of proteinase inhibitors in the process of development. Although their function in plants is obscure, several roles have been proposed including the control of protein hydrolysis and resistance to bacteria and insects (62). It is important that the extent of their

distribution in plants be examined further if their physiological roles are to be established.

It has been shown that fertilization of the ovum requires the presence of a serine proteinase supplied by the spermatozoan. In the control of this enzyme, there may be a potential method of contraception. One demonstration of this possibility is the injection of proteinase inhibitor into the vagina before copulation to prevent fertilization. A great many questions of safety and efficacy remain to be answered before this method could be applied to the control of human reproduction (64). Another benefit which proteinase inhibitors may eventually lead to concerns the development of malignancies. When normal cells are transformed by cancer-producing viruses or by chemical carcinogens, a trypsin-like enzyme is found to be associated with the cell surface (65). The proteolytic enzyme is found in many types of human and animal cancer cells, but is not found in normal cells. The serum of cancer patients, but not that of healthy persons, contains an inhibitor of the enzyme developed by the host in response to the tumor. By blocking the trypsin-like enzyme, the inhibitor can retard the growth and spreading of cancer cells. In cell cultures, low concentrations of

trypsin inhibitors almost totally prevent the growth of transformed cells. Perhaps proteinase inhibitors will be found that can depress the growth rate of cancer cells sufficiently for the immunity to destroy them more quickly than they grow.

Current Research of Barley Proteinase Inhibitors

Barley is a relatively winterhardy and drought resistant grain which generally matures more rapidly than other grains and is widely distributed. It contains about 10-13% protein (66). Cereals generally have a low content of amino acids such as lysine, methionine, threonine, and valine which are essential for monogastric animals. Hiproly barley is a naked cultivar discovered by Swedish workers and has been shown containing high levels of protein and high content of lysine in protein (67). Hiproly with its high protein content provides more of the essential amino acids than any commercially grown cereal grain (68) (see Tables 2 and 3). Waxy Compana barley is a high starch barley and will soon be commercially available for animal diets. It was used here as a comparative study. It was found that lysine, threonine, valine, methionine, isoleucine, alanine, glycine, and aspartic acid are higher

Table 2. Amino Acid Composition of Barley

	Hiproly	Waxy Compana
Protein Content	19.8 gm/100 gm of seed	13.5 gm/100 gm of seed
Lys.	4.2 gm/100 gm of protein	3.2 gm/100 gm of protein
His.	2.2	2.2
Arg.	4.6	3.8
Asp.	7.2	6.8
Thr.	3.2	3.1
Ser.	3.6	3.7
Glu.	25.5	28.8
Pro.	11.6	13.2
Gly.	3.7	3.0
Ala.	4.7	3.0
Cys.	1.0	1.5
Val.	5.2	4.9
Met.	2.0	1.5
Ilu.	3.6	3.4
Leu.	6.5	6.4
Tyr.	2.2	2.5
Phe.	5.3	6.3

Table 3. Nutritional Values of the Proteins of Cereal Grains (Egg Protein as Reference) (68)

Essential amino acid	Egg-reference pattern	Wheat	Oat	Barley Normal	Barley Hiproly
		E/T values[a]			
	3.22	1.99	2.38	2.19	2.17
		A/TE values[b]			
Isoleucine	129	122(95)[c]	102(79)	105(81)	104(81)
Leucine	172	213	194	197	197
Lysine	125	82(66)	110(88)	111(89)	124
Tyrosine and phenylalanine	195	243	220	208	222
Cystine and methionine	107	196	107	94(88)	84(79)
Threonine	99	93(94)	86(87)	97	92(93)
Tryptophan	31	41	42	40	41
Valine	141	150	139	148	147

a - Grams essential amino acids per g total N

b - Milligrams specific amino acid per g of total essential amino acids

c - Values in parentheses are A/TE for specific amino acid/A/TE for egg-reference pattern × 100. The lowest value under a commodity shows the first limiting amino acid and gives a chemical source

in Hiproly, whereas cysteine, glutamic acid, proline are low (67). The protein content of Hiproly was 19.8% of the seed, and the protein contained 4.2% lysine. A normal value is 12.5% protein and 2.9% lysine content (68).

There are three types of inhibitors in barley grains (8); trypsin inhibitor (69), Aspergillus orizae proteinase inhibitors and endogenous proteinase inhibitors (70). Only the trypsin inhibitor has been isolated, purified and its properties investigated (69). Kirsi found that proteinase inhibitors also accumulated in young barley rootlets in high concentration and then disappeared (8). This data implied that the inhibitors were probably synthesized in the meristems and then utilized for growth and development.

In germinating barley, all inhibitory activity disappeared from the endosperms within four to five days after the onset of germination (71). In barley, both endosperms and embryos contained trypsin inhibitor, while the highest trypsin inhibitory activity was found in embryo (72). The inhibitors likely do not have any role in the general matabolism of differentiated vegetative tissues. Most probably, their functions are related to the resting

state or germination. The physiological function of barley inhibitors is as obscure as that of other seed inhibitors.

Few general hypotheses have been put forth to explain the presence of inhibitors in seeds (69). According to one hypothesis, the inhibitors affect endogenous seed proteinases in addition to trypsin and so protect the seed from autolysis during the resting stage. According to another hypothesis, seed trypsin inhibitors inhibit microbial proteinases as well, and their function is to protect the seeds from proteolysis due to micro-organisms. However, barley trypsin inhibitor is totally inactive against all the endogenous proteolytic enzyme and microbial proteinases tested. Another possible explanation involves the endozooic dispersal of seeds. A surprisingly large number of plants, including several leguminosae and gramineae, are at least occasionally distributed by animals that eat fruits or whole plants and excrete viable seeds (73). The presence of inhibitor in seeds in high concentration would certainly, under some conditions, increase the percentage of the seeds passing unharmed through the alimentary tract.

RESEARCH OBJECTIVES

The main purpose of this study was to detect and isolate the proteinase inhibitors in the barley seeds of two different varieties; Hiproly and Waxy Compana.

The specific objectives are listed as follows:

1. to establish high yield extraction procedures.
2. to develop isolation procedures using affinity chromatography with insolubilized trypsin and chymotrypsin.
3. to characterize the general and specific properties such as amino acid composition, molecular weight and stability toward various denaturants.

MATERIALS AND METHODS

Enzyme Assays

Trypsin. The amount of active trypsin in solution was determined either from the rate of catalysis of a specific substrate or by direct titration of its active site. One unit of trypsin activity was defined as the amount of trypsin catalyzing the transformation of one mM of p-toluenesulfonyl-L-arginine methyl ester (TAME) per minute at 25° and pH 8.1 in the presence of 0.05 M $CaCl_2$.

The titrimetrical determination of trypsin activity was based on the method described by Hummel (74). The substrate used was .005 M of TAME with 0.05 M $CaCl_2$ at pH 7.5. Assays were performed with a Radiometer pH stat at 25° with stirring and under nitrogen. As hydrolysis of substrate by trypsin proceeds, 0.05 N NaOH was added automatically to maintain the pH at 8.1. The rate of addition of base was an indication of trypsin activity.

Inhibitor activity was assayed by incubating inhibitor solution (0.1 ml) for five minutes with 0.02 ml of trypsin solution (1 mg/ml) with 0.1 ml of 0.1 M Tris·HCl pH 7.5 buffer. After incubation, 0.1 ml of the mixture was introduced to 10 ml of the 0.005 M TAME solution in the pH stat. The inhibition was determined by comparison

of the rate of base addition with that in the absence of inhibitor. The volume of inhibitor solution assayed was adjusted, as necessary, to provide measurable inhibition (when using 0.02 ml trypsin).

Chymotrypsin assays. For chymotrypsin, the substrate used was N-acetyl-L-tyrosine ethyl ester (ATEE) at an initial concentration of 0.01 M. The assay solution contains .01 M $CaCl_2$ and 5% ethanol to increase the solubility of the substrate (without affecting the assay significantly). Chymotrypsin inhibition was assayed titrametrically using the pH stat.

Elastase assay. Elastase hydrolyzes peptide bonds on the COOH side of amino acids bearing uncharged non-aromatic side chains, principally alanine, glycine and serine (75). This specificity difference is the basis for an assay using N-benzoyl-L-alanine methyl ester (BAME) as a specific substrate which has been reported by Kaplan and Dugas (76). In principle, it is identical of the use of TAME and ATEE to assay specifically for tryptic and chymotryptic activities, and the rate of hydrolysis of BAME may be conveniently followed either spectrophotometrically or by titration in a pH stat. Here the pH stat was used.

Carboxypeptidase B assay. The method employed to measure carboxypeptidase B activity is based on the difference spectra of hippuric acid relative to hippuryl-L-arginine (77). When the absorbancy of a 0.001 M solution of hippuric acid in 0.025 M Tris pH 7.65 containing 0.1 N NaCl was measured against a blank consisting of a 0.001 M solution of hippuryl-L-arginine in the same buffered salt solution, a broad peak was observed in the ultraviolet region with a maximum 254 nm. The hydrolysis of one micro-mole of substrate causes an increase in absorbancy of 0.12 units (76).

Carboxypeptidase A assay. The method employed to measure carboxypeptidase A activity is a differential spectral assay similar to that outlined previously for carboxypeptidase B, except the carboxypeptidase A substrate N-benzoylglycyl-L-phenylalanine (hippuryl-L-phenylalanine) was used in place of the carboxypeptidase B substrate (78).

Pepsin assay. The most widely used assay method for pepsin activity is that developed by Anson (79). Acid-denatured hemoglobin is the substrate at pH 1.8 and

37°, and the release of cleavage products that are soluble in 3% trichloroacetic acid is measured spectrophotometrically at 280 nm.

Isolation and Purification of Barley Inhibitors

Extraction. Barley seeds of Hiproly and Waxy Compana were ground with a CRC micro-mill for about two minutes to give a fine powder. Part of the powder was defatted with a soxhlet apparatus using a chloroform:methanol mixture (2:1, vol:vol). The powder was extracted with 0.05 M Tris·HCl containing 0.01 M $CaCl_2$, .01 N NaCl and 0.01 M ascorbate, pH 7.5 buffer. The extraction was carried out 24 hours with stirring under nitrogen in the cold room. The ratio of volume of extract solution to gm of ground seed was 5:1.

After extraction, solid debris was removed by centrifugation. The solid debris was extracted again with the same buffer about two hours; the supernatant was collected and combined with the initial extract. The pH of extract was adjusted to 4.5 with 6N HCl. Figure 1 shows the extraction scheme used; if any precipitate appeared after adjustment of the pH to 4.5, it was removed by centrifugation.

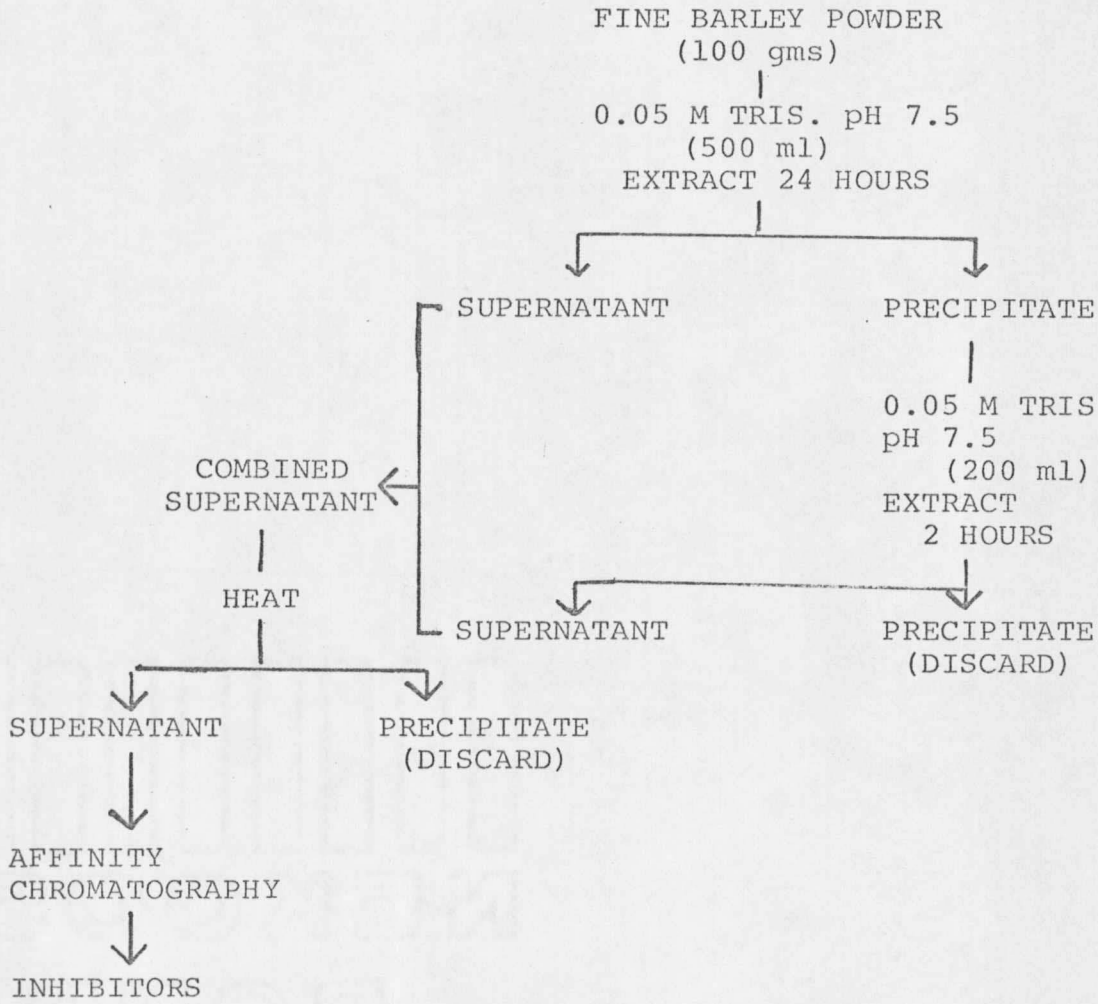

Figure 1. Extraction and isolation procedures of trypsin and chymotrypsin inhibitors from barley.

Heat precipitation. In the case of trypsin inhibitors, the crude extract was heated in a boiling water bath for 15 minutes. After cooling to room temperature, the precipitated protein was removed by centrifugation. For the preparation of chymotrypsin inhibitor, the crude extract was heated for 15 minutes in a 50° water bath. After cooling the extract to room temperature, the precipitated protein was removed by centrifugation. The resulting clear supernatants were used for subsequent absorption onto insoluble enzyme-sepharose.

Affinity Chromatography

Preparation. The preparation of this insoluble trypsin (or chymotrypsin) sepharose was done before extraction of barley inhibitors and was stored in the refrigerator. Coupling of trypsin (or chymotrypsin) to Sepharose 4B was based on the method described by Cuatrecasas (80). The activation of the sepharose was carried out in 200 ml beaker containing a pH electrode, thermometer and a magnetic stirrer. Sepharose 4B was washed on a coarse fritted glass filter with water to remove azide and sucked dry. The damp resin (60 ml) was suspended in 25 ml of water and CNBr solution (3.75 gm

dissolved in 5.0 ml of water) was added. The pH was rapidly adjusted to 11.0 and maintained at that pH by addition of 6 N NaOH and the temperature of the solution was kept at 20° by adding ice periodically.

After 10 minutes, the rate of addition of NaOH slowed down. The mixture was immediately filtered by suction on a coarse sintered glass funnel and washed quickly with two liters of ice water and two liters of cold 0.05 M sodium borate buffer pH 9.0. Filtration and washing took only about five minutes.

A solution of 1.0 gm of trypsin (or chymotrypsin) in 0.05 M sodium borate buffer pH 9.0 containing 0.01 M $CaCl_2$ was prepared just before the sepharose activation and kept at 2°. The activated sepharose was promptly added to this solution and the mixture was stirred gently in the cold room overnight. After coupling, the trypsin sepharose (or chymotrypsin sepharose) was filtered by suction with a sintered glass filter and washed with three liters of 0.05 M sodium borate pH 9.0 containing 0.01 M $CaCl_2$ and three liters of 0.5 N NaCl containing 0.01 M $CaCl_2$. The insolubilized trypsin (or chymotrypsin) sepharose was stored at 4° in a small amount of 1.2 mM HCl containing 0.01 M $CaCl_2$.

Adsorption of inhibitors. The trypsin (or chymotrypsin) sepharose was added to the crude inhibitor solution at room temperature. The pH of the mixture was adjusted to 7.5, and the mixture was stirred gently for about 15 minutes. The sepharose was collected by suction filtration and washed with a pH 7.5, 0.05 M Tris buffer containing 0.1 N NaCl, and 0.01 M $CaCl_2$ until the $O.D._{280}$ of the effluent was less than 0.05 units. The insoluble sepharose trypsin (or chymotrypsin) -inhibitor complex was then poured into a chromatography column for subsequent elution.

Dissociation of inhibitor complex. The enzyme-proteinase inhibitor complex was dissociated by treatment with a low pH buffer. The column was eluted using 0.05 M β-alanine containing 0.1 N NaCl, 0.01 M $CaCl_2$ pH 2.5 as elute buffer. Fractions were collected with a fraction collector; the flow rate of the column was maintained at 31 ml/hr with a Milton Roy piston pump. After collection, the $O.D._{280}$, pH and inhibitory activity of each fraction tube was measured.

Dialysis and lyophilization. Those fractions possessing inhibitory activity were collected and put into

cellulose tubing. Dialysis against water was continued for 24 hours with changes every two hours. The retentate was centrifuged (if necessary) and lyophilized.

Characterization Studies

Heat stability.

A. Two mg of barley inhibitor was dissolved in 2 ml of pH 7.5, 0.05 M Tris•HCl buffer. The solution was incubated at different temperature ranging from 50° to a boiling water (93° C) bath for 15 minutes. After incubation, aliquots were examined for remaining inhibitory activity.

B. Two mg of barley inhibitor was dissolved in 2 ml of 0.05 M Tris•HCl pH 7.5 buffer. The sample was incubated for varying lengths of time in a boiling water bath. At different time intervals, aliquots were removed and the residual inhibitory activity was determined.

Molecular weight determination. Molecular weight determination of inhibitor using gel filtration is described by Fischer (81) and Determann (82). A 1.5×1000 cm column of sepharose G-75 (fine grade) was prepared by the method illustrated Lathe and Ruthven (83). Fully hydrated gel particles were decanted several times to remove the

fines and degassed by placing under vacuum until evolution of dissolved air ceased. Packing was done at room temperature by filling the column partially with water solution into which a portion of gel slurry was poured. Another column was placed on the top, and the entire gel slurry was introduced and allowed to settle under gravity. Two mg samples of barley inhibitor and known molecular weight proteins including ovalbumin (45,000), chymotrypsinogen (25,000), ribonuclease A (13,680), insulin (dimer)(11,000) and blue dextran were dissolved separately in 1 ml of water. Each protein was filtered through the column, using water as eluting solution. A flow rate of 31 ml/hr was maintained and five minute fractions (2.6 ml) were collected. After collection, the optical density of each fraction was read at 280 nm against water.

pH stability. Inhibitor samples (1 mg) were incubated with different buffers (glycine•HCl, Tris•HCl and $NaHCO_3$) ranging from pH 2-11 for five minutes at room temperature. Aliquots were removed and inhibitory activity was determined using TAME or ATEE assay.

Carbohydrate content. Carbohydrate content in the inhibitor sample was determined by the method of

Dubois (84). Different concentrations of inhibitor and sugar standard (glucose) were treated with 1 ml of 89% phenol after mixing with 5 ml of concentrated sulfuric acid, and the solution was kept in the 30° water bath for 10 minutes. The absorbance at 480 nm and 490 nm was determined for pentoses and hexoses, respectively. The amino acid analyzer was used to detect the possible presence of amino sugars, i.e., glucosamine and galactosamine.

Absorption spectra. The ultraviolet absorption spectrum of the barley inhibitor in water (1 mg/1 ml) was determined with a recording Varian Tectron 635 spectrophotometer.

Amino acid analysis. One mg of inhibitor sample was hydrolyzed with 2 ml of 6 N HCl in a sealed, evacuated tube in a 110° oil bath for 24 hours. The analysis was carried out by the method of Spackman et al. (85), using a Beckman-Spinco 120 C amino acid analyzer equipped with a Infortronic CRS 110 A digital intergrater. Results were calculated using a computer program described by Hapner and Hamilton (86).

Isoelectric focusing. Isoelectric focusing was done using LKB produker ampholine mixture (pH 3-10) in polyacrylamide analytical disc gel columns (6.5×0.64 cm). Tubes containing the gel-ampholine column were placed in the electrophoresis tank with 0.2% sulfuric acid in the anodic compartment and 1% ethanol-amine in the cathodic compartment. A current of 1 mA/gel was maintained by slowly increasing the voltage to a maximum of 350 V. Complete focusing required from 2-4 hours, and visualization was accomplished with 12% trichloroacetic acid (TCA). A sample of myoglobin was normally included in a separate tube in order to visually determine when focusing was completed.

Disc gel electrophoresis. Basic (pH 8.3) disc gel electrophoresis was carried out in polyacrylamide gel by the method of Davis (87). Protein samples containing sucrose were applied to the top of the gel, and a current of 2 mA/tube was applied for about 2 hours. Bromophenol blue was used as tracking dye and electrophoresis terminated when the disc of the tracking dye was seen to approach the lower end of the running gel. The bands were visualized with 12% TCA.

Pepsin susceptibility. One mg of inhibitor was dissolved in 1 ml of Tris·HCl pH 7.5 buffer, and the pH was adjusted to 2.0 by adding HCl. The solution was incubated with pepsin (0.02 mg) for 4 hours in 37° water bath. Aliquots were taken to determine the residual inhibitory activity.

Carboxypeptidase A and carboxypeptidase B susceptibility. Inhibitor sample (1 mg) was dissolved in 1 ml of 0.05 M Tris·HCl pH 7.5 buffer. The inhibitor solution was incubated with carboxypeptidase solution (0.02 mg) at 37° for 4 hours. After incubation, remaining inhibitory activity was measured.

Elastase susceptibility. Inhibitor sample (1 mg) was dissolved in 1 ml of 0.05 M Tris·HCl pH 7.5 buffer. After 4 hours incubation with elastase (0.02 mg) in a 37° water bath, residual inhibitory activity was determined.

Chemical Modification of Barley Inhibitors

Oxidation with performic acid (88). Performic acid reagent was prepared by mixing 0.5 ml of 30% hydrogen peroxide and 9.5 ml of 99% formic acid in a closed container at 25° for 2 hours. Two mg of inhibitor were dissolved

in 2 ml of performic acid reagent with 1 ml of 99% formic acid and 0.2 ml methanol. The mixture was cooled in an ice bath (-5° C) for 4 hours. The reaction solution was diluted with 400 ml water and immediately lyophilized.

Reduction and alkylation (98). Two mg of inhibitor was dissolved in 2 ml of 6 M guanidine•HCl containing 0.5 M Tris and 0.002 M EDTA pH 8.1 solution. The solution was kept in 50° water bath for 30 minutes to denature the protein fully. Ten molar excess of dithiothreitol was added and the tube was flushed with nitrogen and maintained at 50° for 4 hours. The solution was then cooled to room temperature, and 10 molar excess of iodoacetamide was added. After 20 minutes in the dark, the mixture was dialyzed and lyophilized immediately.

Active Site Determination of Trypsin Inhibitor

A. Modification of arginine by 1,2 cyclohexanedione (89)

Five mg of trypsin inhibitor was dissolved in 2 ml of 0.2 N NaOH; a ten fold excess of 1,2 cyclohexanedione (over the calculated arginine content) was added. The solution was kept at room temperature for three hours,

then neutralized with 1 N HCl, dialyzed against water and lyophilized.

B. Modification of lysine using citraconic anhydride (97)

Trypsin inhibitor (6 mg) was dissolved in water (2 ml) and the pH was adjusted to 8.2. Aliquots of citraconic anhydride were added to the stirred solution. The reaction proceeded at room temperature and pH 8.2 was maintained by addition of 5 N NaOH. When the addition of citraconic anhydride was completed, the reaction mixture was allowed to stir at room temperature for 2 more hours at pH 8.2, then the solution was dialyzed against water and lyophilized.

REAGENTS

Chemicals used in this study are listed below. All additional chemicals were reagent grade and water was distilled and deionized by a Barnstead deionizer.

Enzymes	Sources
Trypsin	Worthington
Chymotrypsin	Worthington
Elastase	Sigma
Carboxypeptidase A	Worthington
Carboxypeptidase B	Worthington
Pepsin	Sigma

Chemicals	Sources
TAME	Nutritional Biochemicals
ATEE	Nutritional Biochemicals
BAME	Sigma
Hippuryl-phenylalanine	Schwarz-Mann
Hippuryl-arginine	Schwarz-Mann
Dithiothreitol	Calbiochem
Iodoacetamide	Aldrich

RESULTS AND DISCUSSION

Presence of Proteinase Inhibitors in Barley

Trypsin and chymotrypsin inhibitors were detected in the aqueous extracts of ground barley seeds. Five grams of ground seeds were extracted with 50 ml of a pH 7.5 Tris·HCl buffer containing 0.1 N NaCl, 0.01 M $CaCl_2$ and 0.01 M ascorbic acid. After two hours at room temperature, the extract was clarified by filtration and centrifugation and aliquots of the supernatant were tested for inhibitory activity against trypsin and chymotrypsin.

The results are shown in Figure 2. Both Waxy Compana and Hiproly barley contained small amounts of trypsin and chymotrypsin inhibitory activity. Trypsin was inhibited to approximately twice the extent of chymotrypsin for a given amount of extract. A varietal difference was also observed in that Hiproly barley consistently had about 25% more trypsin and chymotrypsin inhibitory activity than did Waxy Compana. Based on total protein present, the trypsin and chymotrypsin inhibition was approximately equal in the two varieties.

From the data in Figure 2, the total amount of proteinase inhibitors present in barley may be calculated. Using assumed molecular weights of 14,000 and 16,000,

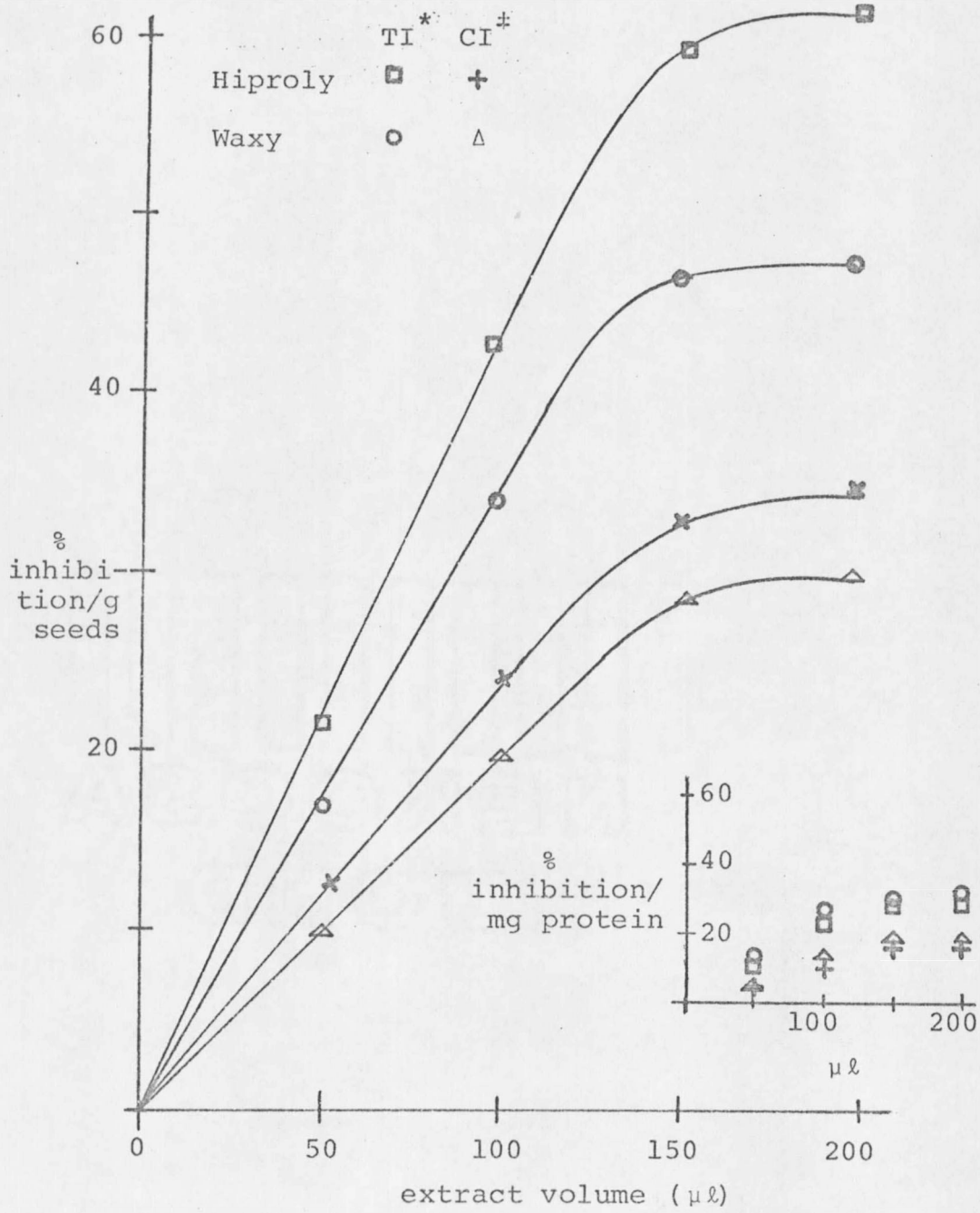

Figure 2. Inhibition of trypsin and chymotrypsin by barley extract.
* - trypsin inhibitor ‡ - chymotrypsin inhibitor

respectively, for the trypsin and chymotrypsin inhibitor, and an assumed binding stoichiometry of 1:1, results in a calculated inhibitor content of Hiproly and Waxy Compana as shown in Table 4. The calculation is shown below.

Table 4. Inhibitor Content in Barley

	Trypsin inhibitor*		Chymotrypsin inhibitor‡	
	g/100 g (seeds)	g/100 g (protein)	g/100 g (seeds	g/100 g (protein)
Waxy Compana	.03	.2[a]	.02	.2
Hiproly	.04	.2[b]	.03	.15
Prikka (69)	.045	.45[c]	N.D.[d]	N.D.
Soybean (6)	2.4	6.0	0	0
Alfalfa (90)	.04	.02	0	0
Sainfoin (91)	.04	.11	0	0

* - All the values calculated are based on commercial trypsin being 60% active

‡ - All the values calculated are based on commercial chymotrypsin being 80% active

a - Based on 14% protein content in Waxy Compana barley

b - Based on 20% protein content in Hiproly barley

c - Based on 10% protein content in Prikka barley

d - Not determined

In assay:

Trypsin content was 9.1×10^{-6} gm, while inhibitory extract was 45.4 μℓ. From Figure 2, 100 μℓ of extract can inhibit 42% of trypsin activity.

The amount of trypsin which was inhibited:

$$9.1 \times 10^{-6} \text{ gm} \times 42\% = 3.8 \times 10^{-6} \text{ gm}$$

The amount of extract which inhibited 3.8×10^{-6} gm of trypsin was 45.4 μℓ. Assuming the stoichiometry of binding is 1:1 allows calculation of the weight of inhibitor present per liter of extract. 3.8×10^{-6} g/45.4 × 10^{-6} ℓ = .0837 gm/l extract.

$$\frac{.0837}{23,800} = \frac{x}{14,000} \qquad x = .05 \text{ gm/l of extract.}$$

The total volume of extract was 800 ml, so the amount of inhibitor present was .05 gm × 80% = .04 gm/800 ml of extract or .04 gm/100 gm of seeds.

The amount of trypsin and chymotrypsin inhibitor in barley is small and approximately equals that found in two legumes, alfalfa (90) and sainfoin (91). Barley contains both types of inhibitors where the legumes contain only trypsin inhibitor. The fact that inhibitors occur only to a small limited extent in the barley (as compared with soybeans) and their susceptibility to degradation by

heat and by pepsin (see below) suggests that their ingestion will not result in nutritional or growth problems.

Factors Affecting the Extraction of Barley Inhibitors

Equal amounts of barley powder were extracted separately with two different buffers (.05 M Na acetate pH 4.9 and .05 M Tris•HCl pH 7.5) under nitrogen, at room temperature for two hours. The inhibitory activity of the supernatant was assayed after centrifugation. The appearance of inhibition is shown in Table 5. It is shown that neutral buffer can solubilize more inhibitor, but the difference is very small. Subsequent preparations were carried out in the Tris•HCl buffer solution.

Both defatted and nondefatted barley powder were extracted individually with Tris•HCl buffer for two hours under nitrogen and in the presence of .01 M ascorbate at room temperature. The inhibitory activity of the extract was determined after centrifugation and the results are shown in Table 6. It can be seen from Table 6 that defatted powder lost some of the inhibitory activity, although it was not significant. This suggests the lipid parts of the barley seeds may contain small amounts of inhibitory materials, or probably the soxhlet solvent

Table 5. Trypsin Inhibition Appearance by the Effect of Extract Buffer.

	Extract volume		
Buffer	25×10^{-3}ml	50×10^{-3}ml	100×10^{-3}ml
Tris·HCl	16±2%	38±2%	60±3%
Acetate	12±1%	28±2%	52±2%

Table 6. Trypsin Inhibition Appearance by the Effect of Defatting Process.

	Extract volume		
Barley seed powder	25×10^{-3}ml	50×10^{-3}ml	100×10^{-3}ml
Defatted	12±2%	32±2%	48±3%
Nondefatted	18±2%	38±3%	60±5%

(chloroform:methanol mixture) can destroy some of the inhibitory materials. The defatting process did not seem to improve extraction of inhibitory activity, so it was not included in subsequent extractions.

The rate of inhibitor appearance in the extract is shown in Figure 3. Barley seed powder was extracted with Tris•HCl buffer, and aliquots of extract were taken at different time periods and measured for inhibitory activity. Inhibitory activity approaches a maximum after two hours extraction and increases no further. It also can be seen that trypsin inhibitory activity is approximately twice the chymotrypsin inhibitory activity.

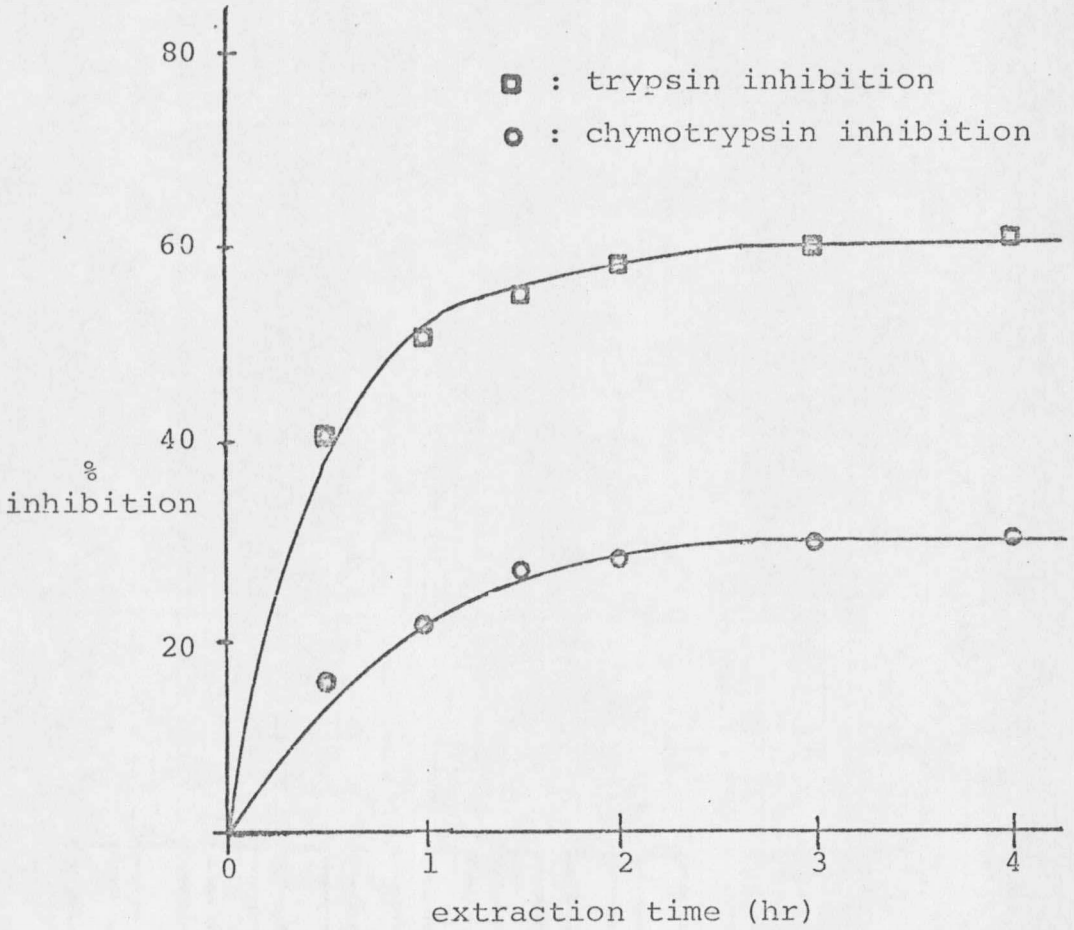

Figure 3. Appearance of inhibitors in Hiproly barley extract.

ISOLATION OF BARLEY INHIBITORS

Dissociation of Endogenous Enzymes-Inhibitors Complex

It is known that some inhibitors form complexes with endogenous enzymes during storage in the seed (6). The result of which should be the dissociation of possible enzyme-inhibitor complex. In order to examine this possibility, the pH of the crude extract was lowered to 4.5 by adding HCl. The inhibitory activity increases slightly if at all. Therefore, no significant increase in yield of inhibitor would be expected by incorporating a low pH step in the isolation procedures. Those increases observed were within the error of the assay procedures and were not significant.

Precipitation of Heat Labile Protein

The crude volume of extract was heated in a boiling water bath (93° C) for 15 minutes. Heat labile protein began to precipitate in about 10 minutes and reached maximum at 13 minutes. After cooling to room temperature and centrifugation, the inhibitory activity of the supernatant was assayed and the results are included in Table 7. Trypsin inhibitory activity of the extract was not affected at all, while chymotrypsin inhibitory activity was about 70% destroyed. It was also found that chymotrypsin inhibitory

Table 7. Effect of Heating on Trypsin and Chymotrypsin Inhibitory Activity.‡

	% of inhibition	
	not heated	heated
Waxy Compana		
Trypsin inhibition	30±4%	33±2% (a) 33±1% (b)
Chymotrypsin inhibition	30±2%	6±1% (a) 30±2% (b)
Hiproly		
Trypsin inhibition	56±4%	58±2% (a) 60±4% (b)
Chymotrypsin inhibition	31±2%	8±2% (a) 30±2% (b)

a - Heated in 93° C water bath for 15 minutes

b - Heated in 50° C water bath for 15 minutes (and up to 4 hours)

‡ - pH 7.5, .05 M Tris·HCl, .01 $CaCl_2$, .01 ascorbate

activity could be fully destroyed if heating continued more than 15 minutes. It was determined, however, that chymotrypsin inhibitory activity was not destroyed by heating at 50° for up to 4 hours. The fact that Mikola (69) isolated no chymotrypsin inhibitor from barley may be due to the heating step employed. He heated the barley extracts in boiling water for 15 minutes. Apparently barley chymotrypsin inhibitor undergoes thermal denaturation between 50° and 95° whereas the trypsin inhibitor is stable.

Adsorption of Barley Inhibitors

Trypsin (or chymotrypsin) can be bound to sepharose resulting in an insoluble active preparation of enzyme-sepharose (80). The trypsin (chymotrypsin) activity was assayed after coupling to the sepharose. It was found that 13.5 mg of trypsin or 15.0 mg of chymotrypsin was bound to one ml of sepharose. Trypsin (chymotrypsin) inhibitor can form a stable complex with trypsin (chymotrypsin) sepharose at neutral pH (14). Insoluble enzyme-sepharose preparations were used to specifically remove the trypsin and chymotrypsin inhibitors from the barley extracts. After the extract was treated with trypsin or

chymotrypsin sepharose at neutral pH for 30 minutes, all of the respective inhibitor activity was removed from the extract. This suggested the inhibitor formed a complex with insoluble trypsin (chymotrypsin) sepharose and was thus removed from solution. During absorption the inhibitor trypsin (chymotrypsin) sepharose complex became stained with amber color. This color could not be washed off with .05 M Tris•HCl buffer. It has been suggested that low pH buffer can avoid the binding of colored materials, but it can also result in trypsin (chymotrypsin) cleavage of susceptible bonds in inhibitors (92). The colored materials are likely noninhibitory, and they might be binding to the sepharose matrix rather than to the insolubilized trypsin. The colored material did not elute from the resin under conditions employed to elute the bound inhibitor.

Dissociation of Sepharose-Trypsin (Chymotrypsin) Barley Inhibitor

The dissociation constant of the inhibitor-enzyme complex is known to be pH dependent (6,93). At the neutral pH range, binding is maximal while no significant binding takes place when the pH is lowered to 2.5. Due to the instability of inhibitor complex at low pH, the inhibitor can be eluted from the sepharose-enzyme complex by

an appropriate buffer. Dissociation was achieved by elution of the complex with .05 M β-alanine pH 2.5 buffer containing 0.1 N NaCl and 0.01 M $CaCl_2$. Figures 4 and 5 show the elution patterns of the inhibitor-trypsin (chymotrypsin) sepharose complex. From Figures 4 and 5, it can be seen that before the addition of pH 2.5 buffer, no inhibitor was eluted from the column. Both trypsin and chymotrypsin inhibitor show one symmetrical peak in the elution pattern, as measured by $O.D._{280}$ and inhibitory activity. The inhibitory fractions were collected, dialyzed and lyophilized. A white fluffy protein resulted. In a single step of affinity chromatography, the inhibitor of barley can be isolated easily. This ease and speed in separating proteinase inhibitor from a crude mixture makes the affinity chromatography a very useful procedure. It was found that the insoluble trypsin and chymotrypsin sepharose did not have durability. Using high salt solutions and low pH buffers, enzyme activity of the enzyme-sepharose preparations could not be regenerated after once used to isolate the proteinase inhibitors. Unknown materials somehow destroyed the trypsin (chymotrypsin) activity of insoluble sepharose-trypsin (chymotrypsin). The

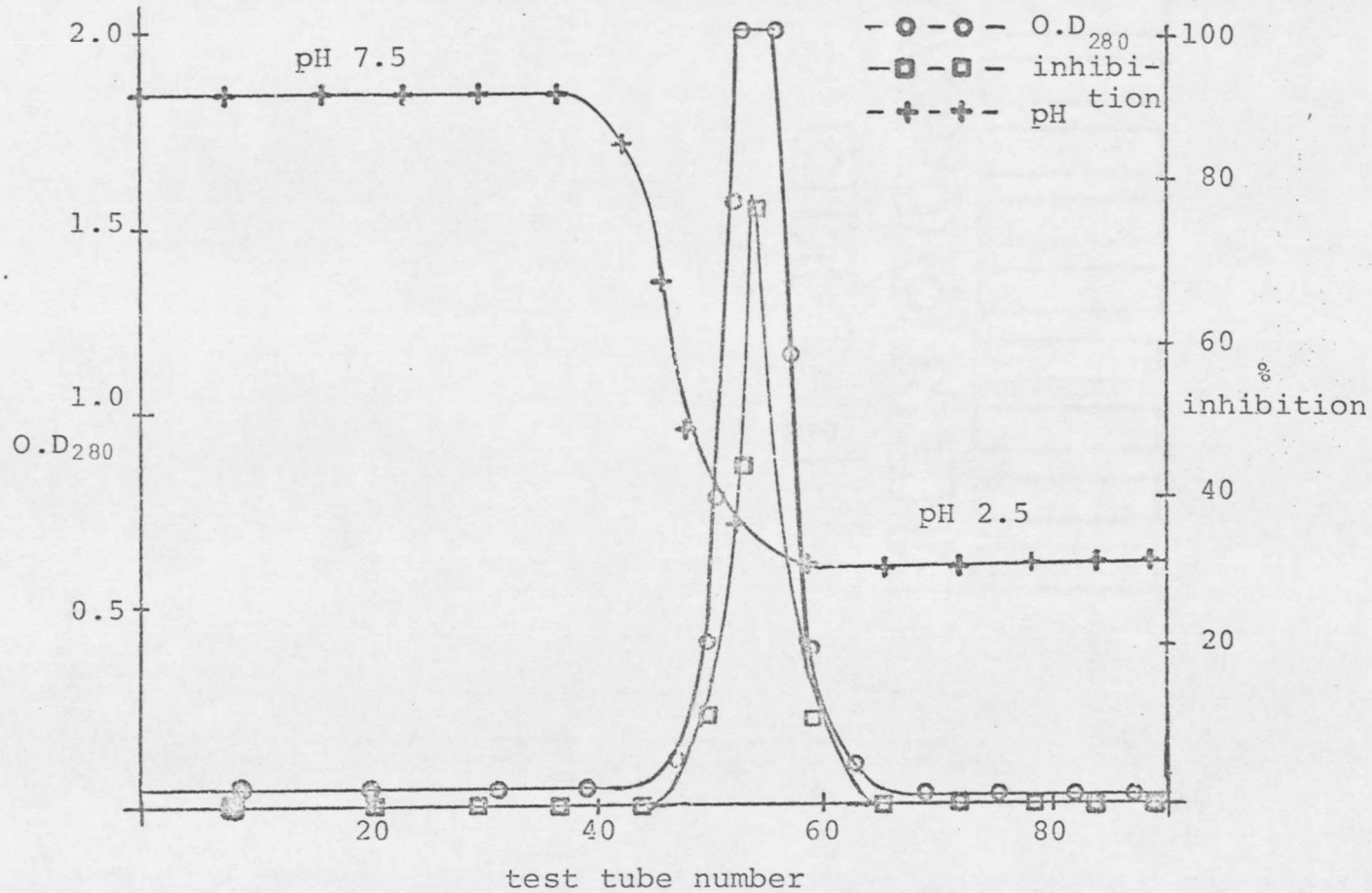

Figure 4. Dissociation of Hiproly trypsin inhibitor-trypsin sepharose complex by column elution with pH 2.5 β-alanine buffer.

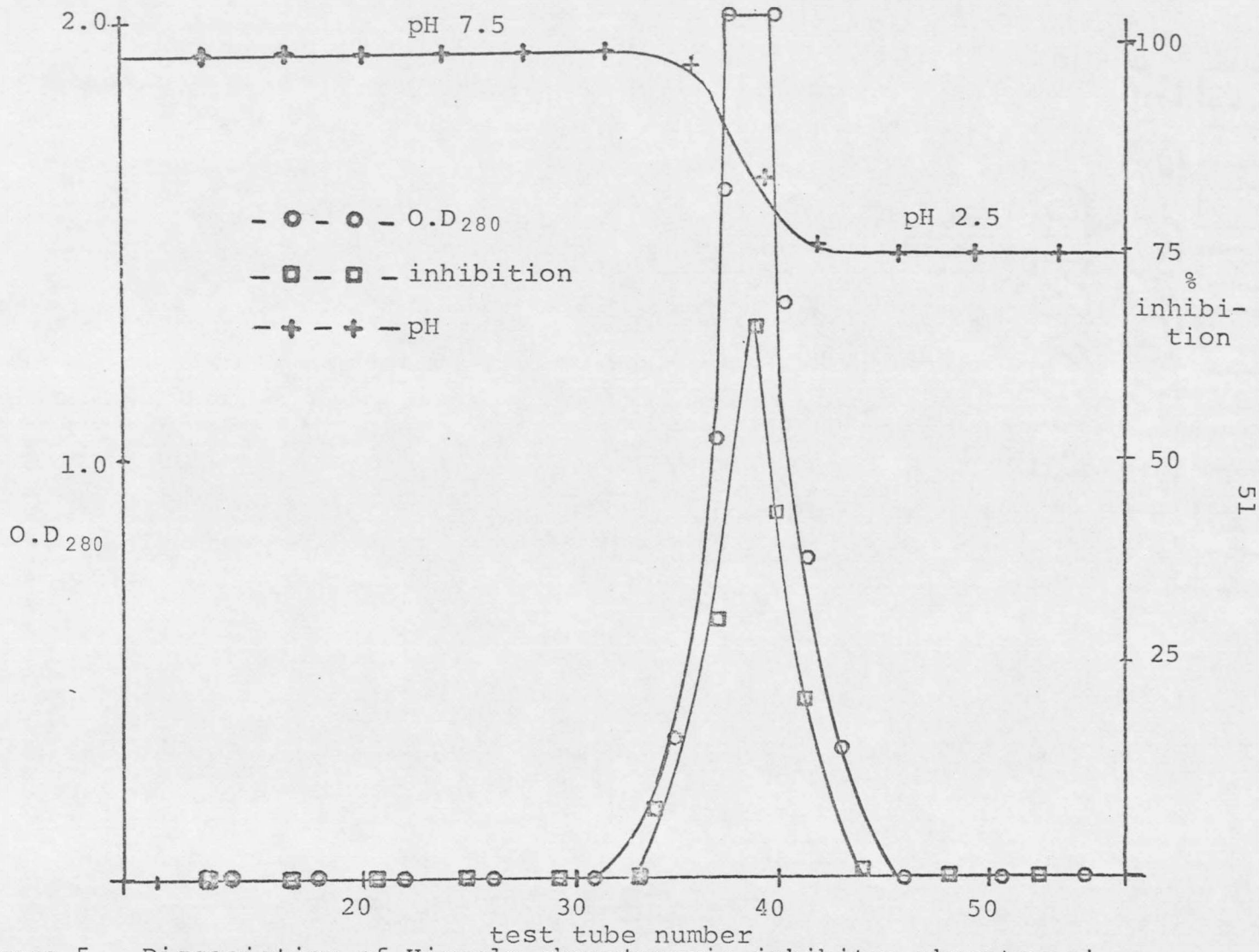

Figure 5. Dissociation of Hiproly chymotrypsin inhibitor-chymotrypsin sepharose complex by column elution with pH 2.5 β-alanine buffer.

conceivable role of barley lectins in inactivating the sepharose preparations was not investigated.

Physical Characterization of Barley Inhibitors

Heat stability. The heat stability of barley inhibitor is shown in Figures 6 and 7. Trypsin inhibitor (2 mg/ml Tris pH 7.5) was stable at 93° C water bath, while chymotrypsin inhibitor (2 mg/2 ml Tris) was about 85% denatured for 15 minutes. Chymotrypsin inhibitor undergoes thermal denaturation at approximately 70°, an observation likely related to the low content of disulfide bonds relative to the trypsin inhibitor.

From Figure 7, it is shown that the trypsin inhibitor still retained 70% of its inhibitory activity after three hours incubation at 93° C water bath. The reason for losing part of the inhibitory activity may be due to denaturation of some minor inhibitor components. There were at least five inhibitor species present in barley (see electrophoresis). The chymotrypsin inhibitor was completely destroyed after one hour incubation in 93° C water bath.

Solubility. The barley inhibitors were soluble in buffers ranging from pH 2 to 12. After five minutes

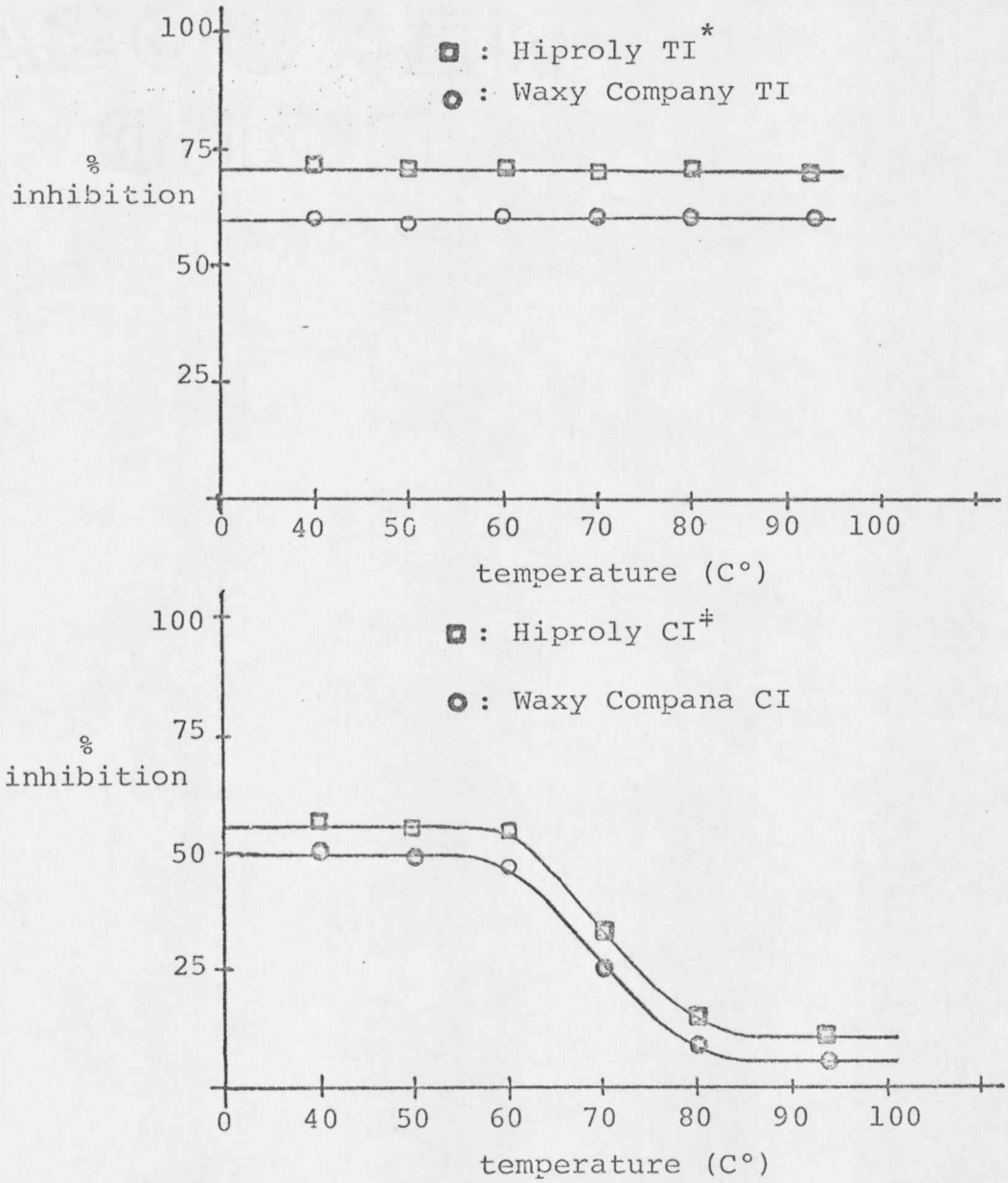

Figure 6. Temperature stability of barley trypsin and chymotrypsin inhibitors.

*TI - trypsin inhibitor

‡CI - chymotrypsin inhibitor

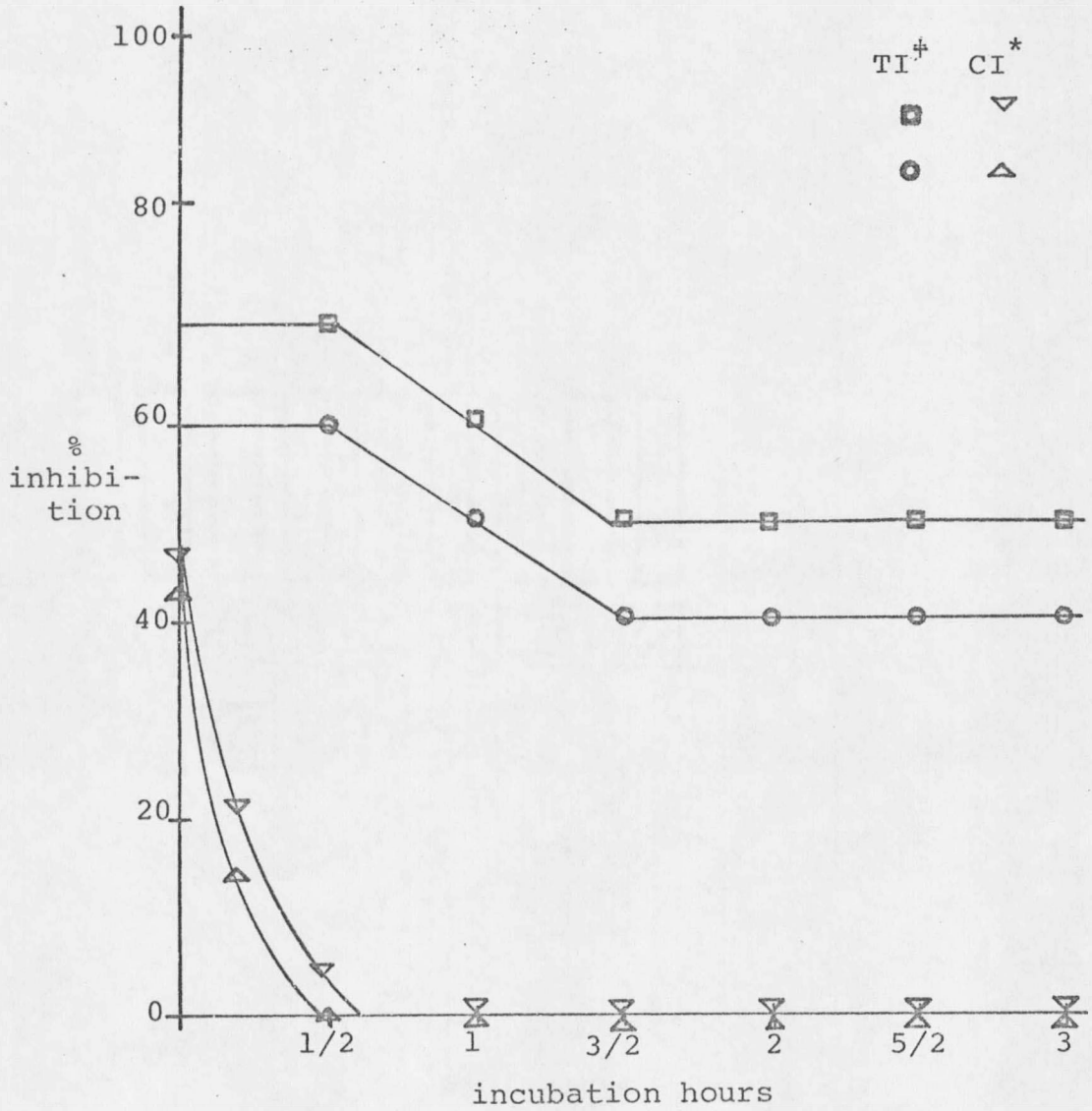

Figure 7. Heat stability of barley inhibitions in 93° C water bath

‡TI - trypsin inhibition

*CI - chymotrypsin inhibitor

incubation at room temperature, no loss of inhibitory activity was detected (see Table 8). This suggests neither inhibitor is irreversibly denatured by extremes of pH.

Table 8. pH Effect on Barley Inhibitor Activity.

	pH		
	pH 2	pH 7.5	pH 12
Trypsin inhibition	58±2%	56±2%	60±3%
Chymotrypsin inhibition	35±2%	40±4%	32±2%

Activities were measured after 5 min. at indicated pH. Other pH values not included for simplicity.

Molecular Weight

The molecular weight determination was based on the method of Fischer and Determan (81,82). Proteins of known molecular weight and inhibitor samples were chromatographed on Sephadex G-75 and the elution volumes were determined. Figure 8 shows the curve relating the elution volume and the log of molecular weight. As seen in Figure 8, it can be calculated the Hiproly trypsin and chymotrypsin inhibitors have molecular weight of 14,180 and 16,620, respectively, while Waxy Compana trypsin and chymotrypsin inhibitor have 15,890 and 18,670. These values

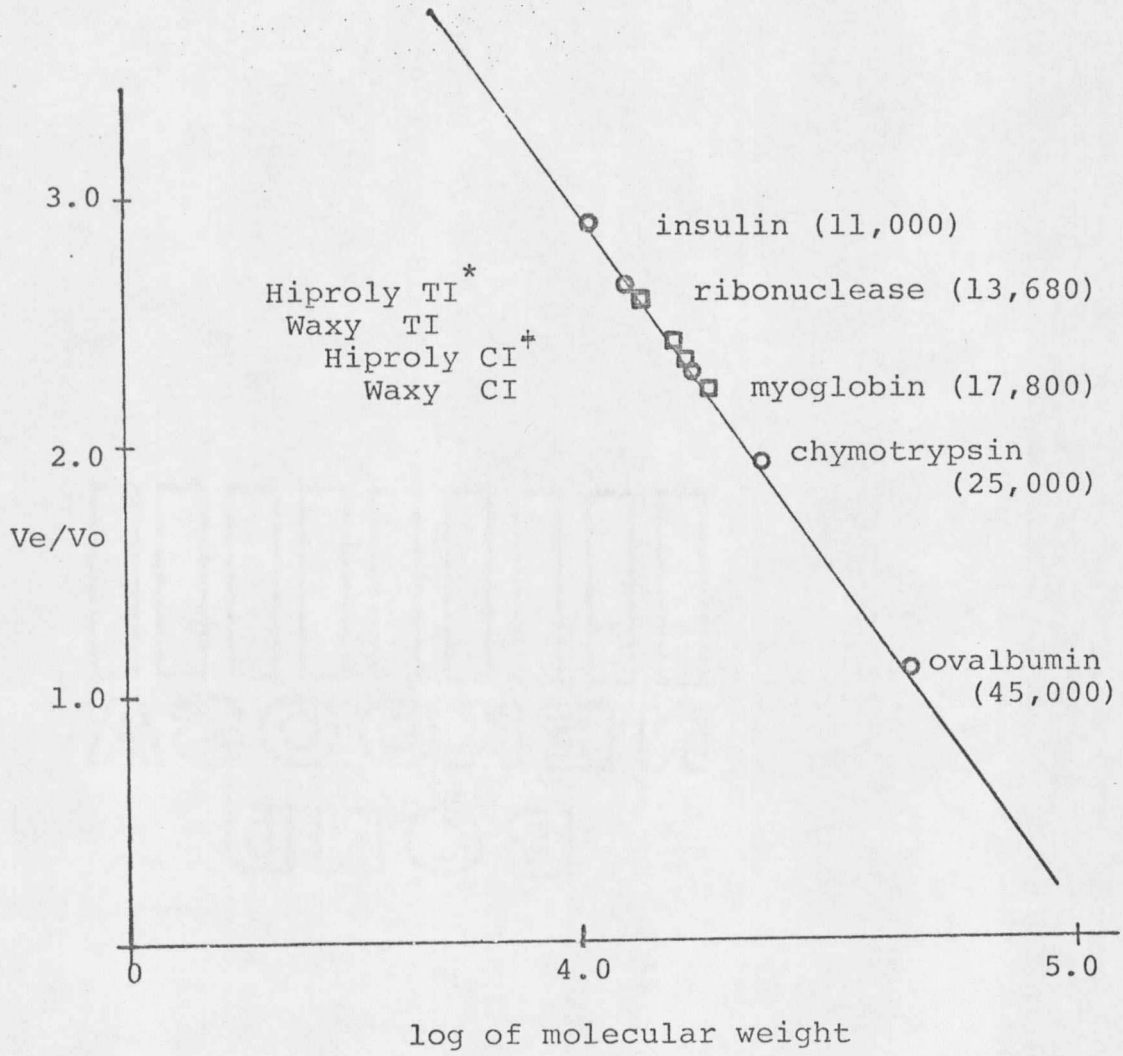

Figure 8. Molecular weight determination using G-75 gel filtration.

* - trypsin-inhibitor, + - chymotrypsin inhibitor

O - known protein, □ - barley inhibitor

were similar to the values from the amino acid composition studies (see Table 9). The molecular weight of trypsin inhibitor from Prikka is known to be 14,400 (69). The molecular weight of trypsin inhibitor from Hiproly (14,180) is the same as Prikka, while Waxy Compana trypsin inhibitor (15,890) is slightly larger than Prikka.

Table 9. Barley Inhibitor Molecular Weight Determination.

	Gel filtration determination	Amino acid analysis
Hiproly trypsin inhibitor	14,180	14,020
Hiproly chymotrypsin inhibitor	16,620	16,300
Waxy trypsin inhibitor	15,890	16,300
Waxy chymotrypsin inhibitor	18,670	18,600

Ultraviolet Spectra

The ultraviolet absorption spectra of barley inhibitors were determined in water solution as shown in Figure 9. The spectra indicate the presence of tyrosine by the observation of absorbance at 280 nm. No obvious tryptophan shoulder at 292 nm was observed. The absence of tryptophane was confirmed by the tryptophane determination

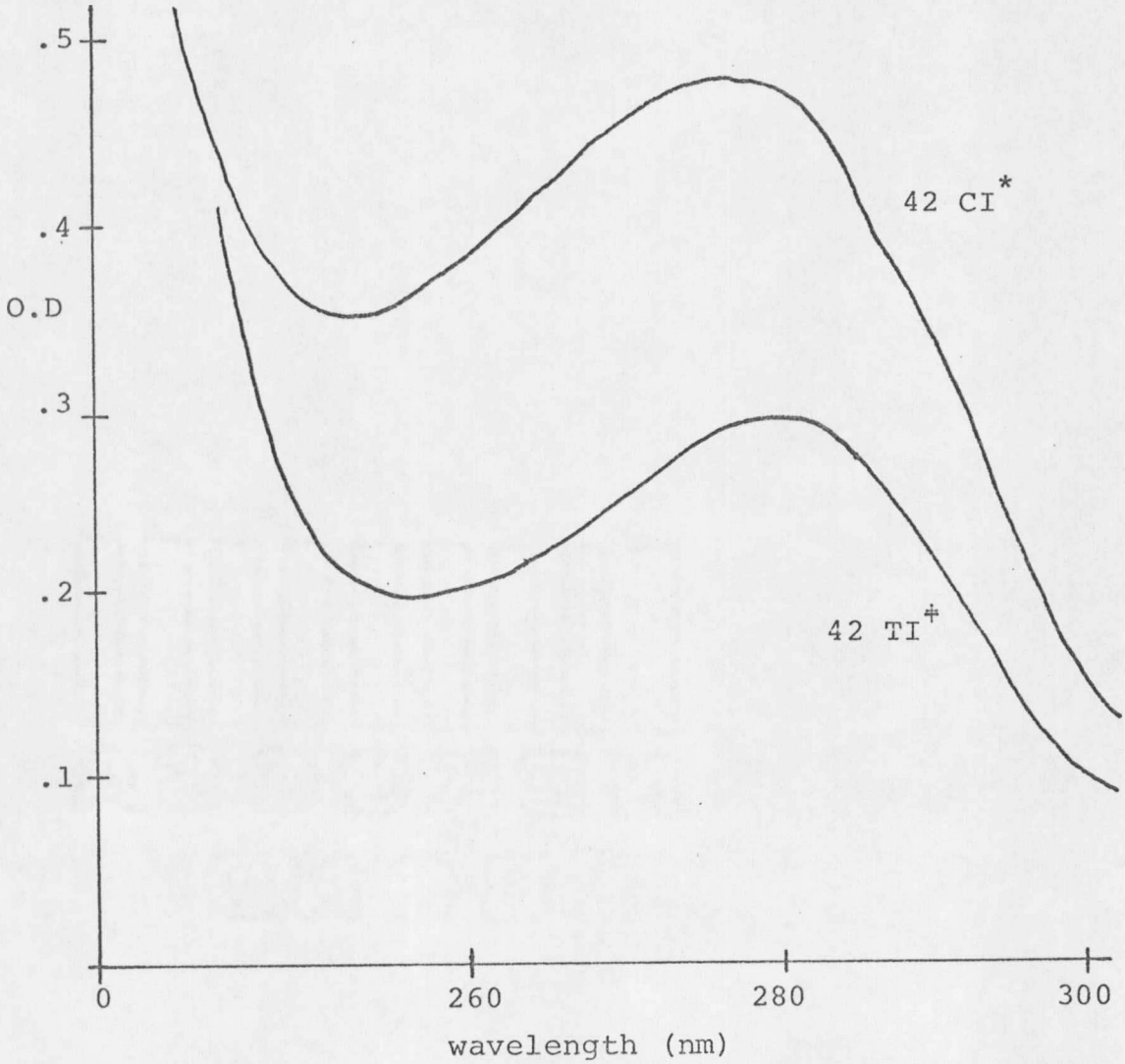

Figure 9. Molecular absorption spectrum of Hiproly barley inhibitor.

*42 CI - Hiproly chymotrypsin inhibitor

+42 TI - Hiproly trypsin inhibitor

using methane sulfonic acid hydrolysis of inhibitor sample (see page 69) followed by amino acid analysis.

The molecular extinction coefficient was calculated from the absorbancy value at 280 nm.

$$A_{280} = E_{280}^{M} \times b \times c$$

A = absorbance at 280 nm

E_{280}^{M} = molar extinction coefficient at 280 nm

b = cell length (cm)

c = concentration (M)

For Hiproly trypsin inhibitor;

$$A_{280} = 0.29 \quad b = 1 \text{ cm} \quad c = 2.88 \times 10^{-5} \text{ M}$$

$$0.29 = E_{280}^{M} \times 1 \times (2.88 \times 10^{-5})$$

$$E_{280}^{M} = \frac{0.29}{2.88 \times 10^{-5}}$$

$$= 8352 \text{ M}^{-1} \text{ cm}^{-1}$$

For Hiproly chymotrypsin inhibitor;

$$A_{280} = 0.47 \quad b = 1 \text{ cm} \quad c = 1.26 \times 10^{-4}$$

$$0.47 = E_{280}^{M} \times 1 \times 1.26 \times 10^{-4}$$

$$E_{280}^{M} = \frac{0.47}{1.26 \times 10^{-4}}$$

$$= 3746 \text{ M}^{-1} \text{ cm}^{-1}$$

The calculated molar extinction coefficient for trypsin was 8352 cm^{-1} M^{-1}. A molar extinction coefficient (280 nm) of 1280 for tyrosine residues and 120 for disulfide bonds was shown by Edelhoch (94). Six tyrosines and five disulfide bonds indicated by amino acid analysis (see amino acid composition) corresponds to the absorption spectrum. The same process was carried out for chymotrypsin inhibitor. The number of disulfide bond (trace amount) and tyrosine residues (1.3) indicated by amino acid analysis (see amino acid composition) was considered to be 1 and 2 which then can correspond to the extinction coefficient calculated from absorption spectrum.

Electrophoresis Patterns of Barley

Figure 10 shows the isoelectric focusing patterns of barley inhibitors in a pH 3 to 10 ampholite gradient.

Trypsin inhibitor shows one major band and four light bands. Chymotrypsin inhibitor shows one major band and four light bands. Seal myoglobin was focused in separate gel to visually indicated when focusing was completed. PI of both inhibitors was estimated in the range of 8∿9. The major band of TI was 8.4 while CI was 8.1. Each band was cut out and soaked in 1 ml Tris buffer and was shown to contain inhibitor activity. Figure 11 shows the barley inhibitors after being subjected to pH 8.3 disc gel electrophoresis. One major band and three light bands were observed for trypsin inhibitor while chymotrypsin inhibitor showed only one band. From this result, it is known that the chymotrypsin inhibitor had different isoelectric point from trypsin inhibitor. It was also found that the locations of bands of both Hiproly and Waxy Compana were the same in trypsin and chymotrypsin inhibitor.

Earlier work has shown both endosperm and embryo of barley seeds to contain proteinase inhibitors (72). Most of the inhibitory activity was found in the embryo. These inhibitors were shown to have the same inhibitory activity but different amino acid compositions. The electrophoretic heterogeneity observed here may be due to the fact that

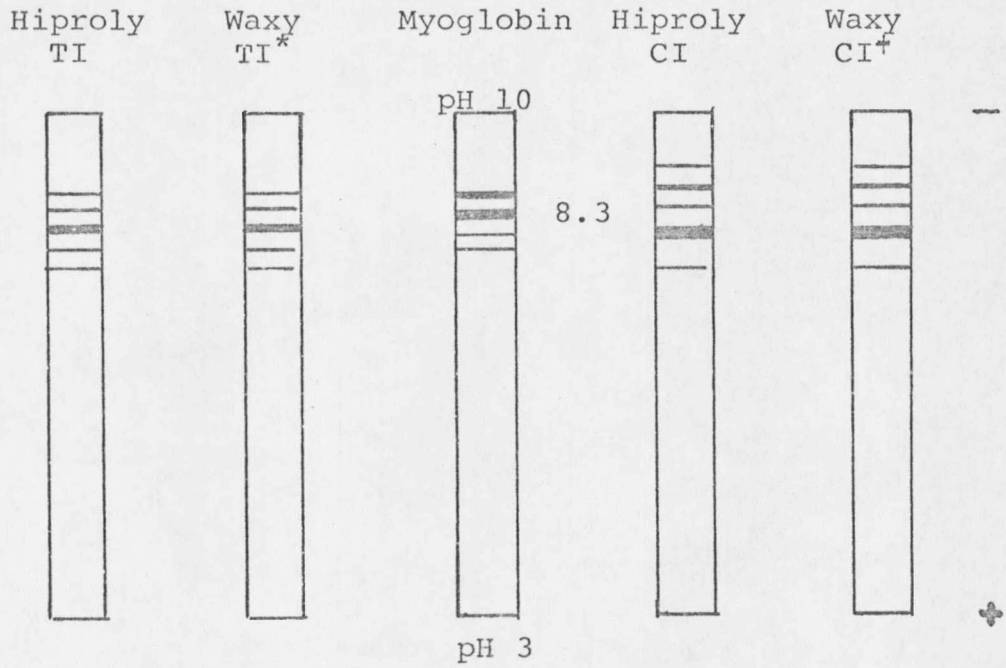

Figure 10. Isoelectric focusing of the barley inhibitors in ampholyte gradient.

*TI - trypsin inhibitor

‡CI - chymotrypsin inhibitor

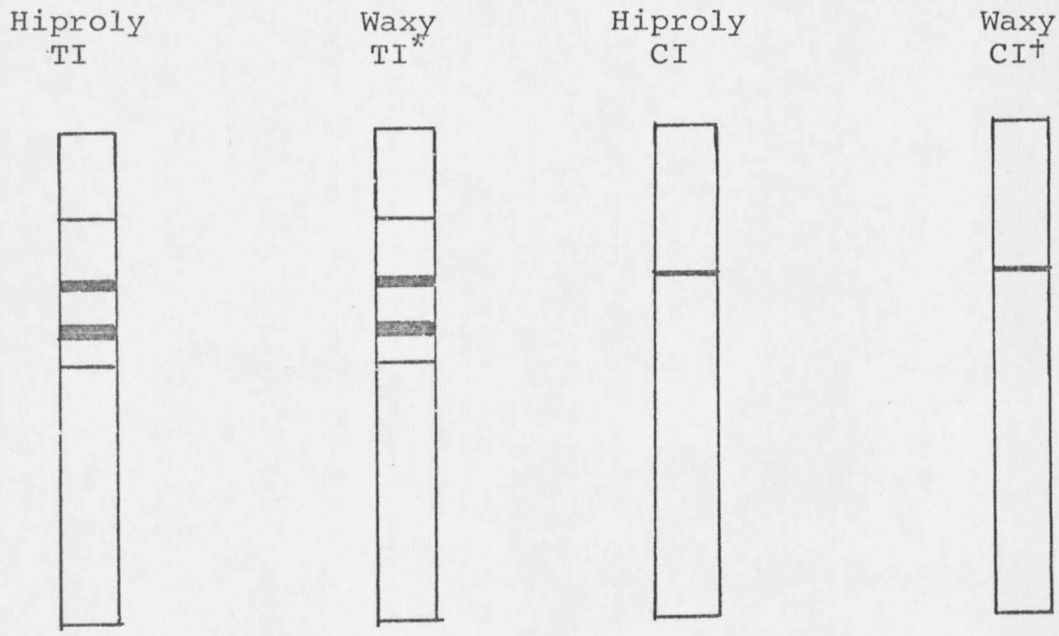

Figure 11. Disc gel electrophoresis patterns of barley inhibitors.

*TI - trypsin inhibitor

‡CI - chymotrypsin inhibitor

whole barley seeds were used in the isolation of proteinase inhibitors.

Enzymatic Characterization of Barley Inhibitors

Barley inhibitor (1 mg/2 ml of Tris·HCl) was incubated separately with pepsin, elastase, carboxypeptidase A and carboxypeptidase B (inhibitor/enzyme = 5:1) at 37° C water bath. After four hours incubation, the inhibitory activity of each solution had disappeared (see Table 10). Figure 12 shows the inhibitory activity of inhibitor was totally destroyed after three hours incubation with pepsin (at pH 2). It was found that inhibitor alone was stable under the same condition.

Table 10. Enzymatic Inactivation Barley Inhibitors.

	Before incubation		After incubation	
	Trypsin inhibition	Chymotrypsin inhibition	Trypsin inhibition	Chymotrypsin inhibition
Elastase	55±2%	35±2%	0	0
Pepsin	55±2%	35±2%	0	0
Carboxy-peptidase A	55±2%	35±2%	0	0
Carboxy-peptidase B	55±2%	35±2%	0	0

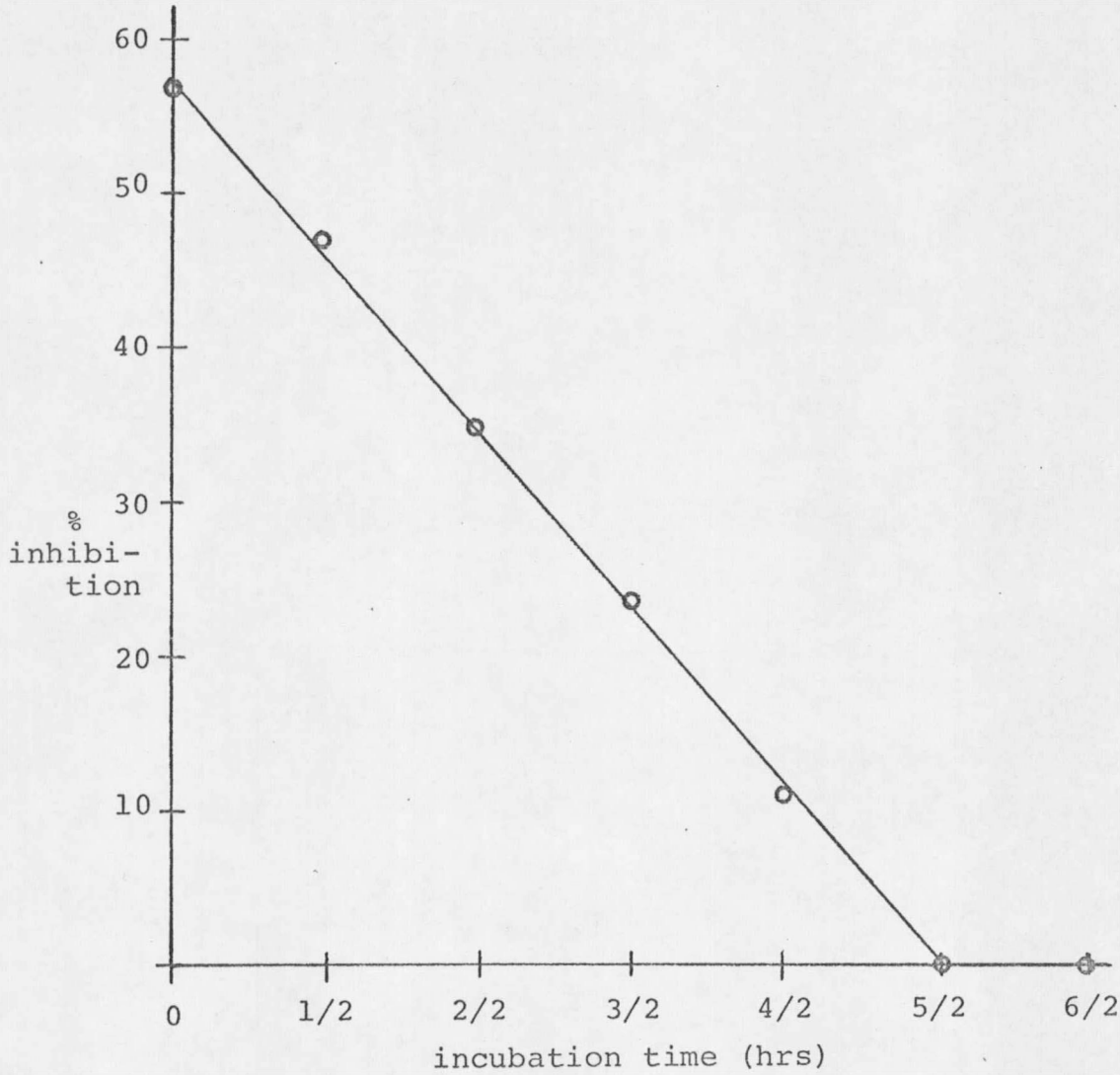

Figure 12. Inhibition of Waxy Compana trypsin inhibitor after incubation with pepsin at pH 1.8 in 37° C water bath.

The evidences of small amounts of inhibitor present in barley seed and the digestibility of inhibitor by proteolytic enzymes indicate barley inhibitors are not responsible directly for any physiological and nutritional effect on animal diet. These results together with the high protein and high lysine content of Hiproly barley should be seriously considered for the animal and human protein resources.

Specificity

There was no cross-inhibition between the trypsin and chymotrypsin inhibitors. Trypsin inhibitor inhibited only trypsin and chymotrypsin inhibitor inhibited only chymotrypsin. No inhibition of pepsin, elastase, carboxypeptidase A or B was observed even at high inhibitor-enzyme ratios (2 μg inhibitor/1 μg enzyme). In fact, both inhibitors served as good substrates for these digestive proteinases and were consequently inactivated as shown in Table 10.

Chemical Characterization of Barley Inhibitors

Carbohydrate content. It is generally known that plant proteinase inhibitors do not contain carbohydrate (6). It has been shown by Mikola (69) that Prikka barley

contains no sugars or amino sugars. The carbohydrate content of barley inhibitor was examined by the method of Dubois (84). Figure 13 shows a standard curve of known sugar concentration and inhibitor samples. No absorbancy change was detected, when varying concentration of barley inhibitor was tested. It indicated that the barley trypsin inhibitor contained no carbohydrate. Chymotrypsin inhibitor was not tested. No amino sugars were detected in the amino acid analysis of the barley inhibitors.

Amino Acid Composition

The amino acid composition of barley inhibitors is shown in Table 11. Hiproly trypsin inhibitor has about 125 amino acid residues, indicating an approximate molecular weight of 14,000. Hiproly chymotrypsin inhibitor has a molecular weight of 16,000 and about 145 amino acid residues.

In Waxy Compana, it was shown that both inhibitors have about 20 more amino acid residues than in Hiproly. The molecular weight of Waxy Compana inhibitors was about 2,000 more than Hiproly barley inhibitors. The amino acid composition suggests that the two trypsin inhibitors and two chymotrypsin inhibitors are closely related. The 20

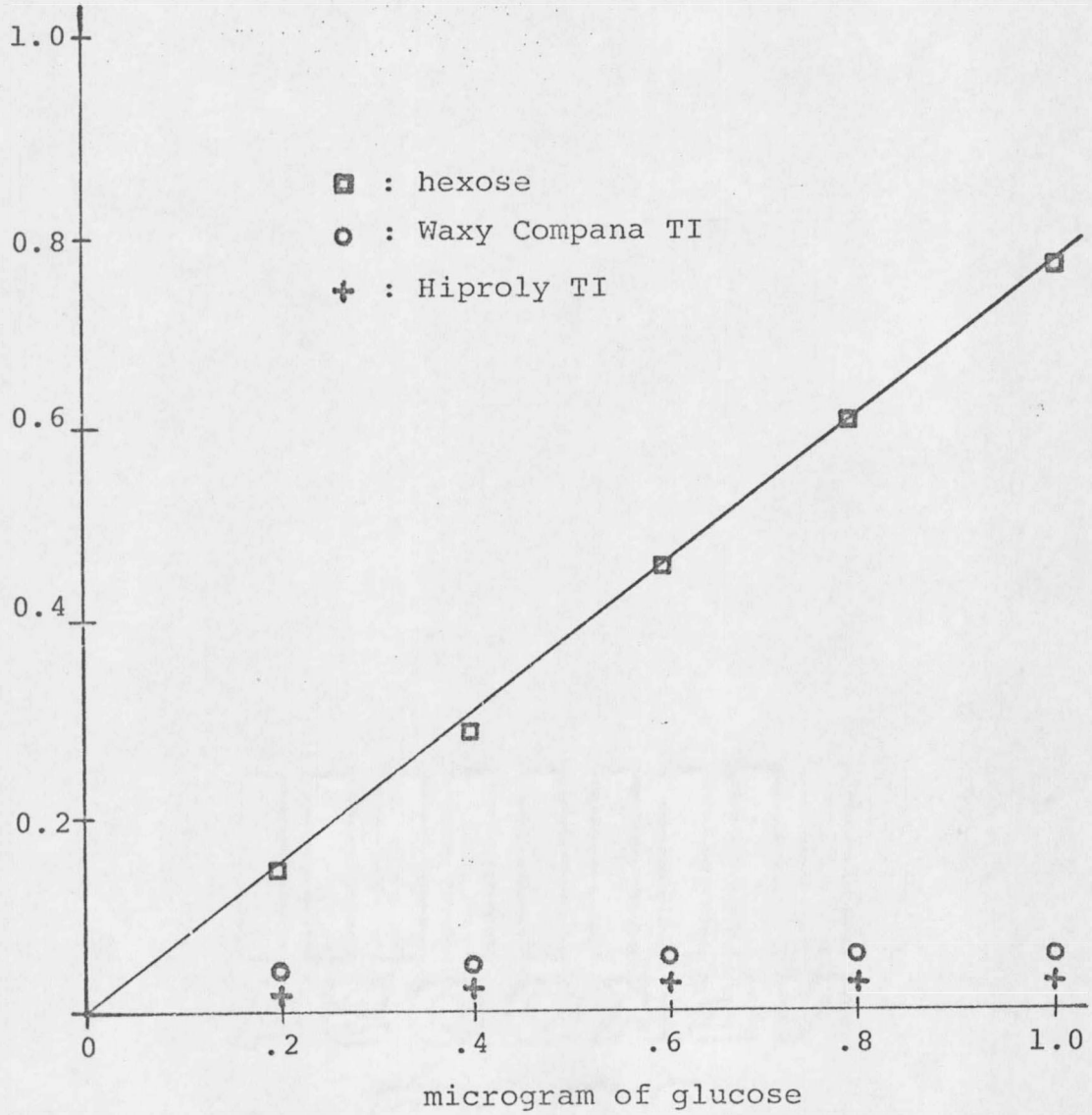

Figure 13. Determination of sugar content of barley trypsin inhibitor.

Table 11. Amino Acid Composition of Barley Inhibitors*.

	Hiproly		Waxy Compana	
	Trypsin inhibitor	Chymotrypsin inhibitor	Trypsin inhibitor	Chymotrypsin inhibitor
Lys.	3.1	13.2	4.1	13.9
His.	3.0	3.0	3.0	3.0
Arg.	9.0	7.0	8.0	8.0
Asp.	9.5	11.8	11.7	13.7
Thr.†	6.1	8.1	6.9	8.6
Ser.†	7.5	9.5	8.9	9.8
Glu.	12.9	19.1	15.2	22.0
Pro.	13.0	9.5	14.0	14.8
Gly.	11.2	13.3	12.4	14.7
Ala.	10.4	10.9	12.4	14.4
½Cys.	9.0	T	11.3	T
Val.	6.2	18.1	7.9	21.3
Met.	2.3	2.3	2.5	1.9∇
Ilu.	5.0	7.5	5.9	8.8
Leu.	8.7	9.4	10.1	8.5
Tyr.	5.5	1.3	6.2	2.3
Phe.	3.0	2.0	3.4	2.7
Trp.Δ	0	0	0	0
recovery	80%	50%	90%	70%
appro. mol. wt.	14,000	16,400	16,000	18,800
residues	125	145	144	168

* - Amino acid composition based on 3 histidine
† - Not corrected for Thr. Ser. destruction
Δ - Methane sulfonic hydrolysis
∇ - As methionine sulfone
T - Trace amount

amino acid difference in each case could be related to the absence of an N or C terminal fragment. The molecular weight determined by amino acid analysis was similar to gel filtration determination (see Table 9).

The determination of tryptophan was based on Liu and Chang's method (95). After 24 hours hydrolysis in methane sulfonic acid, no tryptophan was detected by amino acid analysis. The half cystine content is about 10 in trypsin inhibitor of both varieties. The half cystine content of chymotrypsin inhibitor was only a trace amount in the standard amino acid analysis. In the oxidized chymotrypsin inhibitor, the cysteic acid content was shown to be 1.3 (since no free sulfhydryl was detected--see page 77), it is reasonable to assign a half cystine content of 2, i.e., equal to one disulfide bond. The tyrosine content in both inhibitors is identical with the amount calculated from absorption spectrum (see page 57).

It is interesting to know the difference in contents of charged amino acid residues (Lys, Arg, Glu, Asp) between chymotrypsin inhibitor (45∿50) and trypsin inhibitor (34∿40). From the fact that barley trypsin inhibitor is stable at 93° C water bath, while chymotrypsin inhibitor is just stable at 50° C water bath. It is possible

that the stability of trypsin inhibitor is mainly contributed by the covalently disulfide bonds, which is not easily destroyed simply by heating. Chymotrypsin inhibitor possibly is stabilized mainly by the electrostatic force between charged groups which can be disrupted by heating.

Amino acid composition of trypsin inhibitors from different varieties is listed on Table 12. It can be seen that the amino acid composition of different varieties is more or less the same in all these varieties, but Waxy Compana is a little different, has about 20 more amino acid residues which may be the N or C terminal fragments. Prikka trypsin inhibitor has three tyrosine residues, while the other two do not. Both varieties do show the typical characteristics of proteinase inhibitor; high content of proline (10% of total residues), and disulfide bonds.

Titration of Trypsin (Chymotrypsin) with Barley Inhibitors

Figure 14 shows the titration curve obtained when trypsin and chymotrypsin concentration was held constant, while inhibitor concentration was increased. It can be seen from Figure 14 that both inhibitors form complex reversibly with enzyme and trypsin inhibitor can form complex

Table 12. Amino Acid Composition of Trypsin Inhibitors From Different Varieties.[a]

	Hiproly	Waxy Compana	Prikka(69)
Lys.	3.1	4.1	2
His.	3.0	3.0	3
Arg.	9.0	8.0	9
Asp.	9.5	11.7	10
Thr.	6.1	6.9	7
Ser.	7.5	8.9	8
Glu.	12.9	15.2	14
Pro.	13.0	14.0	11
Gly.	11.2	12.4	10
Ala.	10.4	12.4	10
½Cys.	9.0	11.3	10
Val.	6.2	7.9	6
Met[b]	2.3	2.5	2
Ilu.	5.0	5.9	5
Leu.	8.7	10.1	9
Tyr.	5.5	6.2	5
Phe.	3.0	3.4	3
Trp.[c]	0	0	3
recovery	80%	90%	-[d]
residue	125	144	127
mol. wt.	14,000	16,000	14,400

a - Based on 3 histidines

b - As methionine sulfone

c - Methane sulfonic acid hydrolysis

d - Not recorded

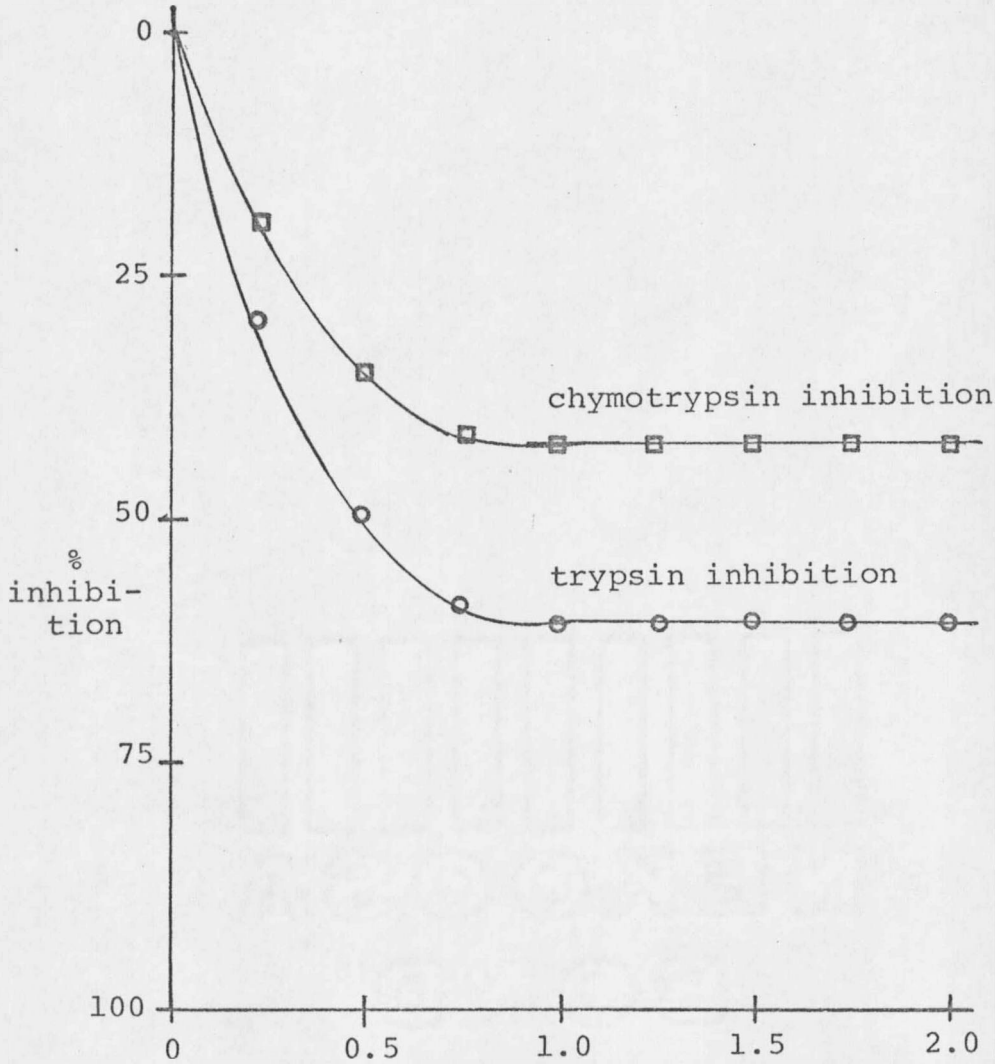

Figure 14. Trypsin and chymotrypsin inhibition in presence of increasing amount of Hiproly barley trypsin and chymotrypsin inhibitor.

with trypsin about twice stronger than chymotrypsin inhibitor can form complex with chymotrypsin. The stoichiometry of 0.8 and 1.2, respectively, for trypsin inhibitor and chymotrypsin inhibitor was extrapolated from the curve.

The dissociation constant can be calulated by using the following equations:

$$T + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} TI \qquad \text{.....(1)}$$

$$K_{diss.} = \frac{k_2}{k_1} = \frac{[T]\ [I]}{[TI]} \qquad \text{.....(2)}$$

TI : concentration of complex

T : concentration of free active trypsin

I : concentration of free inhibitor

At the point of 50% trypsin inhibition from Figure 14, the trypsin inhibitor concentration was 3.6×10^{-6} M while the trypsin concentration was held constant at 5.1×10^{-7} M. The amount of enzyme-inhibitor complex can be determined by 50% of trypsin inhibition.

	trypsin	trypsin inhibitor
initial concentration.	5.1×10^{-7}	3.6×10^{-6}
amount of complex.	2.6×10^{-7}	2.6×10^{-7}
free amount	2.5×10^{-7}	3.3×10^{-6}

The equilibrium dissociation constant can be calculated using the concentration values above to be 3.3×10^{-6} M at pH 8.0. The dissociation constant of chymotrypsin inhibitor was also calculated to be about 5.3×10^{-6} M at pH 8.0. These values are about 200-300 times larger than alfalfa trypsin inhibitor dissociation constant (1.6×10^{-8}M) at pH 8.0 (90).

Chemical Modification of Barley Inhibitors.

Active site determination of trypsin inhibitor. It is experimentally found that all trypsin inhibitors tested can be divided into two classes: 1) lysyl inhibitors which are rapidly inactivated by lysine modifying reagents, and 2) arginyl inhibitors which are inactivated by arginine modifications but are generally unaffected by lysine modification.

The nature of the active site of barley trypsin inhibitor was investigated by using chemical reagents which

specifically modify either arginine or lysine amino acid side chains. Arginine was modified using 1,2-cyclohexandeione (89) and lysine residues were reacted with citraconic anhydride (97). The results are shown in Table 13. Trypsin inhibitory activity appears to be lost upon treatment of the inhibitor with either of the two "active site" reagents. All inhibitory activity was lost after modification with 1,2-cyclohexanedione and most of the activity was destroyed with the lysine reagent. The lysine inactivation was reversible and after exposure to low pH for several hours essentially all the inhibitory activity returned. This result is confusing in that essentially all activity was destroyed by both reagents. If two types of barley inhibitor were to exist, it might be expected that each reagent would destroy a portion of the inhibitory activity proportioned to the amount of lysine or arginine-type inhibitor present. Since both reagents destroyed all activity, it must concluded that formation of the inhibitor-trypsin complex is interfered with by modification at lysine or arginine. It follows that both lysine and arginine are included in the binding of inhibitor to trypsin or that the two residues are located sufficiently close to one another that modification of either

precludes complex formation. There are several examples of trypsin inhibitors which contain both lysine and arginine in the binding region of the inhibitor. Perhaps barley trypsin inhibitor is another.

Table 13. Barley Trypsin Inhibitor's Inhibition Before and After Modification with Cyclohexanedione and Citraconic Anhydride.

	Native trypsin inhibitor	Trypsin inhibitor modified with 1,2-cyclohexanedione	Trypsin inhibitor modified with citraconic anhydride
% of inhibition	54±2%	0	0
control†	54±2%	50±2%	52±1%

† - Treated with base only

Free Sulfhydryl Group Determination

The inhibitor was reacted with DTNB (dithio-2-nitrobenzoic acid) to detect any free sulfhydryl groups (98). After 30 minutes reaction, no absorbancy change was observed. This result indicated that the inhibitor had no free sulfhydryl groups. It is similar to most of the other inhibitors in this regard (6).

Oxidation with Performic Acid

The inhibitor was oxidized with performic acid according to the method described by Hirs (88), followed by determination of the amino acid composition of the modified protein. The amino acid composition of oxidized inhibitor was compared with the native inhibitor.

The oxidized trypsin and chymotrypsin inhibitors had no inhibitory activity remaining. This is the expected result assuming that the disulfide bonds are essential in maintaining the functional configuration of the molecule. Cysteic acid was found in both oxidized inhibitors. Assuming the absence of free thiol group, the presence of the cysteic acid was taken as an indication of disulfide content. Likewise, methionine content was confirmed by the presence of methionine sulfone. Table 14 gives the yields of cysteic acid and methionine sulfone in the oxidized samples.

The presence of just one disulfide bond in the chymotrypsin inhibitor was confirmed by the detection of 1.7 residues of cysteic acid. In this respect, the chymotrypsin inhibitor is quite different from the trypsin inhibitor which has 5 disulfide bonds. This difference in disulfide

content may be related to the heat lability observed for the chymotrypsin inhibitor.

Table 14. Cysteic Acid and Methionine Sulfane Recovery From Oxidized Hiproly Inhibitors.

	Before oxidation		After oxidation	
	TI	CI	TI	CI
1/2 cystine	9.0	trace	0	0
cysteic	0	0	8.5	1.7
methionine	1.8	1.7	0	0
met sulfone	0.5	0.6	1.5	1.7

Reduction and Alkylation

The inhibitor was reduced and alkylated with iodoacetamide by the method of Konigsberg (98). No inhibitory activity was found in the products. This result suggests that disulfide bonds of both inhibitors are required for the inhibitory activity. It also tends to confirm the presence of a disulfide bond in the chymotrypsin inhibitor, as indicated by the oxidation studies.

SUMMARY

The content of trypsin and chymotrypsin inhibitor in barley is small; .03-.04 g/100 gm seeds for trypsin inhibitor and .02-.03 g/100 gm seeds for chymotrypsin inhibitor. The extraction of inhibitor is not significantly affected by changing the buffer pH or using defatted seed powder. After extracted with .05 M Tris·HCl buffer pH 7.5 for two hours, the maximum inhibitory activity was reached, and no further increase was observed for another 20 hours.

Both the tryptic and chymotryptic inhibitory activity were detected in the crude extract. By using insoluble trypsin and chymotrypsin sepharose, trypsin inhibitor and chymotrypsin inhibitor was isolated. Since the trypsin inhibitor and chymotrypsin inhibitor have different heat stabilities, the isolation procedures were carried out differently. For trypsin inhibitor, the precipitation of heat labile protein was carried out at 93° C water bath while for chymotrypsin inhibitor, it was carried out at 50° C water bath.

The barley trypsin inhibitor was stable in 93° C water bath while chymotrypsin inhibitor was totally denatured after one hour incubation. Both inhibitors were soluble from pH 2 to 12 and still retained inhibitory

activity. The whole barley grain contained five species of trypsin and chymotrypsin inhibitors. Each inhibitor was specific for trypsin or chymotrypsin. No inhibition on other proteinase was observed, the inhibitor was inactivated by the proteolytic enzymes pepsin, elastase, carboxypeptidase A and carboxypeptidase B.

Molecular weights were 14,000 and 16,400 of Hiproly trypsin and chymotrypsin inhibitor; 16,000 and 18,800 for Waxy Compana trypsin and chymotrypsin inhibitor. Gel filtration was also used for molecular weight determination, and similar results were observed 14,180 and 16,620 for Hiproly trypsin and chymotrypsin inhibitor; 15,890 and 18,670 for Waxy Compana trypsin and chymotrypsin inhibitor. Both inhibitors lacked tryptophan.

The trypsin inhibitors contained 5-6 disulfide bonds. While chymotrypsin inhibitor showed only one disulfide present. This difference in disulfide content was related to the heat stability difference between trypsin inhibitor and chymotrypsin inhibitor. The small amount of inhibitors present in barley plus their heat sensitivity and susceptibility to digestive enzymes indicates that barley inhibitor will pose no related physiological or

or nutritional problems. The proteins should be easily digestible in the alimentary tract of animals.

Electrophoresis showed 5 species of inhibitors were present in the whole barley grain powder. No carbohydrate or amino sugar was associated with either inhibitor. No free sulfhydryl groups were detected in either inhibitor. The extinction coefficient and absorption spectrum of inhibitor was determined and confirm the disulfide bond and tyrosine contents in each inhibitor.

Oxidized and reduced inhibitors lost all inhibitory activity. The active site of the trypsin inhibitor was susceptible to both lysine and arginine reagents, suggesting both types of residues are located in the active site region of the inhibitor.

Some of the binding equilibrium properties of both inhibitors were examined. The stoichiometry of both inhibitor to enzyme was determined to be near one which is generally seen in plant proteinase inhibitors. The trypsin inhibitor showed twice affinity to trypsin than chymotrypsin inhibitor toward chymotrypsin. The dissociation equilibrium constant of enzyme-inhibitor complex was determined about 200 times bigger than some constant in alfalfa and soybean inhibitors.

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Isolation and characterization of trypsin and chymotrypsin inhibitors from barley

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