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
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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	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.

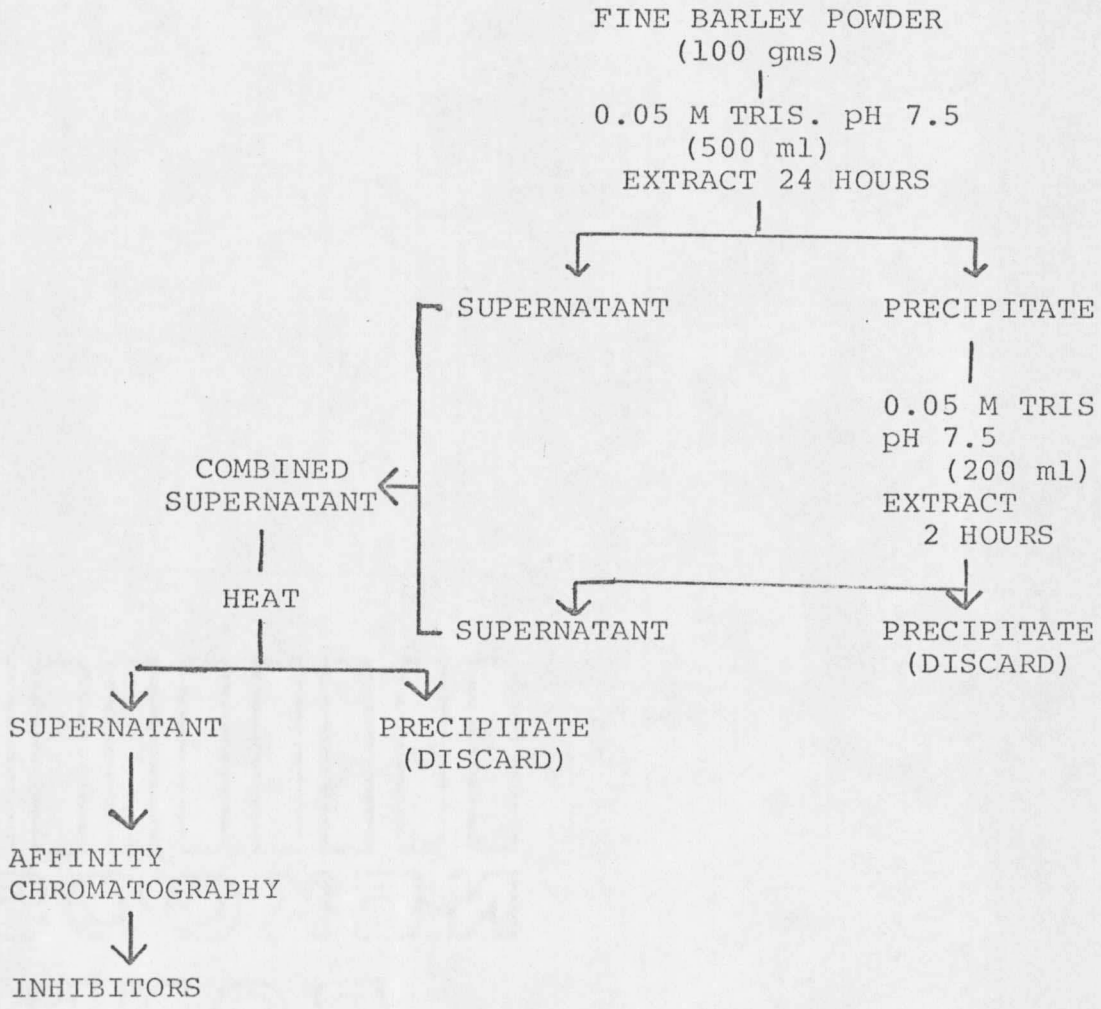

Figure 1. Extraction and isolation procedures of trypsin and chymotrypsin inhibitors from barley.