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**STUDIES OF ENZYME INHIBITORS AND ENDOCHITINASE  
IN SEEDS OF JOB'S TEARS (Coix lachryma-jobi)**

**A thesis presented by**

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(M.Sc. Fortaleza, Brazil)**

**Submitted for the degree of Doctor of Philosophy  
in the University of Durham**

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**Department of Biological Sciences, March 1994**



**- 7 JUN 1994**

This thesis is entirely the result of my work. It has not been submitted for a degree in this or any other university, and is not being submitted for any other degree

To my daughter,

Juliana

Studies of Enzyme Inhibitors and Endochitinase from  
Seeds of Job's Tears (Coix lachryma-jobi)

Studies of the purification, characterization and primary structure of protein inhibitors of trypsin and  $\alpha$ -amylase from seeds of Job's Tears (Coix lachryma-jobi) were undertaken.

The major trypsin inhibitor from seeds of Coix was purified by heat treatment, fractional precipitation with ammonium sulphate, ion-exchange chromatography, gel filtration and preparative reversed-phase HPLC. The complete amino acid sequence was determined by analysis of peptides derived from the reduced and S-carboxymethylated protein by digestion with trypsin, chymotrypsin and the S.aureus V8 protease. The polypeptide contained 64 amino acids with a high content of cysteine. The sequence exhibited strong similarity with a number of Bowman-Birk inhibitors from legume and cereal seeds.

A protein inhibitor of locust gut  $\alpha$ -amylase was purified from seeds of Coix using ammonium sulphate precipitation, affinity chromatography on Red Sepharose and reversed-phase HPLC. It consisted of two major isomers, each a dimer of two identical or closely similar subunits of  $M_r$  about 26 400. These two isomers also had very similar amino acid compositions. The major isomer showed no inhibitory activity against amylases from other sources: human saliva, porcine pancreas, B. subtilis, A. oryzae and barley malt. The manual DABITC/PITC method was used to determine about half of the amino acid sequence of the major isoform. This showed a high degree of similarity with previously reported sequences of endochitinase enzymes from several species (tobacco, potato, barley, bean). Endochitinase activity was demonstrated by following the release of radioactivity from [ $^3\text{H}$ ] chitin. As far as can be ascertained from the literature this is the first characterization of a plant protein with activity as an enzyme and as an enzyme inhibitor.

Preliminary molecular studies were also carried out, including the isolation and in vitro translation of mRNA fractions from developing seeds of Coix.

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## ABBREVIATIONS

ATP	- adenosine 5' triphosphate
BAPNA	- N-benzoyl-DL-arginine-p-nitroanilide
BSA	- bovine serum albumin
cdNA	- complementary DNA
Ci	- curie
CP	- creatine phosphate
C-terminal	- carboxy terminal
cv.	- cultivar
dH <sub>2</sub> O	- distilled water
DABITC	- 4.N,N-dimethylaminoazobenzene-4'- isothiocyanate
DABTH	- dimethylaminoazobenzene thiohydantoin
DABTZ	- dimethylaminoazobenzene thiazolinone
DEAE	- diethylaminoethyl
DNA	- deoxyribonucleic acid
DTT	- dithiothreitol
EDTA	- ethylenediamine tetraacetic acid
g	- grammes or force of gravity
GTP	- guanosine 5' triphosphate
h	- hours
Kb	- kilobases
l	- litres
min	- minute
ml	- millilitre
M <sub>r</sub>	- relative molecular mass
N-terminal	- amino terminal

PAGE	- polyacrylamide gel electrophoresis
PCK	- phosphocreatine kinase
PITC	- phenylisothiocyanate
RNA	- ribonucleic acid
RP-HPLC	- reversed-phase high performance liquid chromatography
RT	- room temperature
rpm	- revolution per minute
SDS	- sodium dodecyl sulphate
SDW	- sterile, distilled, deionized water
TEA	- Tris/EDTA/acetic acid
TEMED	- N, N, N, N', tetramethylene diamine
TRIS	- 2-amino 2-hydroxymethyl propane-1,3-diol
Triton X-100	- iso-octylphenoxypolyethoxy ethanol
v	- volume

CHAPTER 1

INTRODUCTION



## 1. General Introduction

Protein inhibitors of enzymes are widespread in nature. They can inhibit a wide range of enzymes such as hydrolases (e.g. proteinases,  $\alpha$ -amylases), oxido-reductases, transferases and lyases (Tashiro et al., 1989; Rele et al., 1980; Hass and Hermodson, 1981; Shivaraj and Pattabiraman, 1981; Tsafaris et al., 1980; Dullo, 1980; Vesely et al., 1977; Goldstein et al., 1973; Wu and Whitaker, 1990; Tashiro et al., 1991; Odani et al., 1986).

Plant proteinase inhibitors have received particular attention. There are numerous reviews concerning their structure, distribution, activity and possible biological roles (Ryan, 1973, 1979, 1981; Birk, 1976; Richardson, 1977, 1981, 1991; Ryan and Walker-Simmons, 1981; Liener and Kakade, 1980; Belitz and Weder, 1990; Xavier-Filho and Campos, 1984; Laskowski and Kato, 1980; Whitaker, 1981, 1983; Garcia-Olmedo et al., 1987; Gatehouse, 1984; Buonocore and Silano, 1986; Rackis et al., 1986; Weder, 1981, 1986; Bode and Huber, 1992). The best known group of these inhibitors are those which affect the activities of serine proteinases such as trypsin, chymotrypsin and subtilisin. During the last decade much attention has also focused on the plant inhibitors of  $\alpha$ -amylase. One striking finding is the structural and evolutionary relationships of different  $\alpha$ -amylase inhibitors with different types of proteinase inhibitors. This interesting finding as well as several other surprising discoveries arose from the fact that the amino acid sequences of many of the

enzyme inhibitors from plants have been determined. These inhibitors have been grouped (as listed in Table I) in ten most likely families (Richardson, 1991).

Since the exact role of the plant enzyme inhibitors still remains unclear, some hypotheses on the physiological functions of them have been suggested, based on their general properties. Thus, they could act as storage proteins or as regulators of endogenous enzymes. On the other hand, for years research has been carried out on chemicals associated with defense against insects and pathogens (fungi, bacteria, viruses). For a long time many chemicals were considered to be "secondary plant products" with unknown functions. Now they are considered to be defensive chemicals which contribute to the resistance of plants against insects or pathogens. These chemicals include complex substances such as antibiotics and alkaloids, as well as proteins such as enzyme inhibitors and lectins (Richardson, 1977, 1991; Xavier-Filho and Campos, 1989; Xavier-Filho, 1992; Ryan, 1990). These proteins may form part of a defense mechanism of PR ("pathogenesis-related") proteins (Pierpoint and Shewry, 1987). Other components of the PR protein complex are enzymes such as chitinases, lysozymes and  $\beta$ -glucanases (Nasser et al., 1988; Metraux et al., 1988; Pierpoint et al., 1990)

This chapter first deals with some important findings of research on protein inhibitors of enzymes, with particular emphasis on plant inhibitors of proteinases and  $\alpha$ -amylases. Then, additional plant proteins, e.g. chitinases, are discussed.

Table I - Plant protein inhibitor families

Family	Monomer $M_r$ (k)	Enzymes Inhibited	Distribution (main sources)
1. Kunitz (STI family)	21-22	Serine proteinases and endogenous $\alpha$ -amylases	Leguminosae Gramineae
2. Bowman-Birk (BBI family)	8-9	Serine proteinases	Leguminosae Gramineae
3. Potato I	8-9	Serine proteinases	Widespread (Leguminosae, Gramineae, Solanaceae, Polygonaceae, yeast and an animal (leech, <u>Hirudo medicinalis</u> )
4. Potato II	6	Serine proteinases	Solanaceae
5. Cucurbit	3	Serine proteinases Hageman factor	Cucurbitaceae
6. Cereal Superfamily	12-13	Serine proteinases and heterologous $\alpha$ -amylases	Gramineae
7. Ragi I-2	9	Serine proteinases and heterologous $\alpha$ -amylases	Gramineae
8. Maize/Thaumatococcus family	22-23	Serine proteinases and heterologous $\alpha$ -amylases	Gramineae Solanaceae
9. Cystatin	12	Sulphydryl proteinases	Gramineae
10. Carboxy-peptidase	4	Metallo-carboxy-peptidases	Solanaceae

## 1.1 Protein Inhibitors of Enzymes

Details of the history, specificity and distribution of the protein inhibitors of enzymes are summarized. Studies on their primary structures and biological roles are also described.

### 1.1.1 History, Specificity and Distribution

#### HISTORY

We can find indications of the occurrence of protein inhibitors of enzymes (from nematodes) in the literature as early as 1878 (Fredericq, 1878). The first report that indicated the presence of such proteins in plants was given by Read and Haas (1938). They recognized that an aqueous extract of soybean (Glycine max) flour inhibited trypsin. Following this discovery, two different trypsin inhibitors in soybean were identified. One was partially purified by Bowman (1944, 1946). The crystallization of a second one and its complex with trypsin was carried out by Kunitz (1945, 1946, 1947a, b, 1949). A little earlier, Chrzaszcz and Janicki (1933) described the presence of protein inhibitors of  $\alpha$ -amylase in wheat, while Kneen and Sandstedt (1943, 1946) reported the existence of inhibitors of  $\alpha$ -amylase in the grain of different cereals.

After these pioneering studies, these proteins have attracted the attention of many scientists working in different fields as animal nutrition, biochemistry, pharmacology and medicine.

## SPECIFICITY

The specificities of plant protein inhibitors of enzymes differ widely and are also highly variable.

In some cases, like the proteinase inhibitors, they can exhibit a very narrow range of specificity, being capable of inhibiting only one or two closely related proteinases. Some of these compounds can inhibit only trypsin but most of them are active against trypsin and chymotrypsin. Sometimes the reactive site of the inhibitor is the same for both enzymes but several other inhibitors were demonstrated to be "double-headed", i. e., containing different reactive sites for the independent inhibition of the two proteolytic enzymes (Odani et al., 1986; Morhy and Ventura, 1987). Sometimes they can inhibit a range of other serine proteinases e. g. elastase (Wilson and Laskowski, 1975; Richardson, 1991). Others additionally inhibit sulphhydryl enzymes (e.g. papain, Kassel and Williams, 1976).

There is no uniform pattern of specificity exhibited in the case of  $\alpha$ -amylase inhibitors from different sources, although inhibition of human pancreatic and insect salivary  $\alpha$ -amylases is commonly found (Warchalewski, 1983). Gutierrez et al. (1990) described the in vitro effects of crude inhibitor preparations from barley and from two wheat species on the  $\alpha$ -amylases of 23 insect pests of agricultural importance. The results suggested a complex pattern of specificity, where the crude inhibitor preparations have differential effects not only among groups of insects (i. e. storage cereal pest vs. others) but also between species within a group and between

developmental stages within species. Inhibition of endogenous  $\alpha$ -amylases is less often reported but has sometimes been described (Mundy et al., 1984; Peruanskiti and Gabsattarova, 1979; Weselake et al., 1983, 1985).

A number of bifunctional plant protein inhibitors have been isolated which are effective inhibitors of enzymes with completely different specificity. An unusual bifunctional  $\alpha$ -amylase/trypsin inhibitor was isolated from seeds of ragi (Indian finger millet, Eleusine coracana (Gaerth) by Shivaraj et al. (1982). Manjunath et al. (1983) purified an  $\alpha$ -amylase/trypsin inhibitor from the same source but this protein had properties significantly different from that of the  $\alpha$ -amylase/trypsin inhibitor reported earlier by Shivaraj et al. (1982). Unusual bifunctional inhibitors have also been identified in barley (an  $\alpha$ -amylase/subtilisin inhibitor, Mundy et al., 1983) and wheat (an  $\alpha$ -amylase/subtilisin inhibitor, Mundy et al., 1984 and an  $\alpha$ -amylase/trypsin inhibitor, Warchalewski, 1987). More recently, Ohtsubo and Richardson (1992) reported the complete amino acid sequence of a bifunctional subtilisin/ $\alpha$ -amylase inhibitor from bran of rice seeds.

Sometimes the interpretation of the published data on the specificity of inhibitors can be complicated. A good example is in the case of the 22 kDa bifunctional maize inhibitor of bovine trypsin and the  $\alpha$ -amylase from Tribolium castaneum beetles, which is ineffective against the  $\alpha$ -amylases from mammalian and bacterial sources (Richardson et al., 1987). Another good example is the many  $\alpha$ -amylase inhibitors from

wheat that are active against mammalian, insect and avian enzymes, but inactive on plant, bacterial and fungal enzymes. In other words, many of these inhibitory proteins have a strong action on an enzyme from one particular source but no action on the same enzyme from a different source. Also, sometimes the investigators have not examined a full range of potentially inhibited enzymes. Consequently great care is necessary in interpreting the published data on the specificity of inhibition (Richardson, 1991).

#### **DISTRIBUTION**

Protein inhibitors of enzymes are ubiquitous and occur in various tissues of microorganisms, plants and animals.

#### **Proteinase Inhibitors**

After Kunitz (1945, 1946) isolated and purified a trypsin inhibitor from soybean (Glycine max), much of the early work was carried out by those interested in animal nutrition who were concerned about the possible deleterious dietary effects resulting from the presence of these proteins in important food plants. Thus, many studies have been done to determine their distribution in the plant kingdom, and in particular their occurrence in those plant tissues commonly used for food. This is why amongst the large number of known inhibitors, those which are components of plant seeds and

tubers have received most attention. The proteinase inhibitors appear to have been most extensively studied in seeds of legumes (Leguminosae family) because of the large number of species which form important sources of food and the high levels of inhibitors which are present (Weder, 1981). They also represent a quantitatively important fraction of the protein found in most cereal grains (Gramineae family, Boisen, 1983). They have been also extensively studied in the Solanaceae family. Tubers of the potato (Solanum tuberosum L.) are a notable source of proteinase inhibitors (Bhat et al., 1981). Especially high concentrations are found in seeds of the Leguminosae (soybean 20 g/kg, Rackis and Anderson, 1964) and of the Gramineae family (2-3 g/kg, Mikola and Kirsi, 1972) but also in tubers from the Solanaceae family (potato 2-3 g/kg, Mikola and Kirsi, 1972). The inhibitor content depends very much on the variety and physiological status of the plant and on the levels of insect infestation or damage (Liu and Markakis, 1987; Richardson, 1977).

The vegetative parts of many plants were also shown to contain inhibitors. Some species of the Leguminosae family were shown to have proteinase inhibitors in their leaves (Hazelwood et al., 1983; Maia, 1982; Abe et al., 1985).

Proteinase inhibitors occur also in vegetables such as cabbage, cucumbers, tomatoes and spinach, and in certain fruits such as apples, banana, avocado, peach, plum and grapes (Belitz and Weder, 1990; Rao, 1991). They have also been detected in bacteria (Ikenaka, 1974), fungi (Matern et al.,

1974) and in algae (Beitz, et al., 1978). Products of secretion like gum arabic and catechu gum obtained from trees of the Leguminosae, were shown to have serine proteinase inhibitory activity (Weder, 1978),

More than one inhibitor has been demonstrated in many plants. Frequently the picture is further complicated by the fact that several of the inhibitor types may exist in the form of a number of closely related "iso-inhibitors" (Bruhn and Djurtoft, 1977; Belitz et al., 1971).

#### $\alpha$ -Amylase Inhibitors

Most of the proteins which show  $\alpha$ -amylase inhibitory activity have been detected in seeds from cereals and legumes. However, they are also found in some fruits and tubers of plants. Some examples of these inhibitors are given in Table II.

They have been reported also in microorganisms (Ashauer et al., 1981; Murao et al., 1980, 1983; Vertesy and Tripier, 1985; Saito, 1982).

#### 1.1.2 Families Defined on Sequence Relationships

##### KUNITZ INHIBITOR FAMILY (STI FAMILY)

Following the crystallization of the trypsin inhibitor from soybean (STI) by Kunitz (see section 1.1.1), its complete amino acid sequence was determined (Koide and Ikenaka,

TABLE II - Protein inhibitors of  $\alpha$ -amylase in plants

Source	Plant Tissue	Reference
<u>Arachis hypogea</u> (peanut)	Seed	Irshad and Sharma (1981)
<u>Avena sativa</u> (oat)	Seed	Elliot and Leopold (1953)
<u>Canavalia ensiformis</u> (jack bean)	Seed	Jaffé <u>et al.</u> (1973)
<u>Cicer arietinum</u> (chick pea)	Seed	Singh <u>et al.</u> (1982)
<u>Colocasia antiquorum</u> (colocasia)	Tuber	Krishna Sharma and Pattabiraman (1980)
<u>Dioscorea alata</u> (yam)	Tuber	Krishna Sharma and Pattabiraman (1982)
<u>Dolichos lablab</u> (field bean)	Seed	Jaffé <u>et al.</u> (1973)
<u>Dolichos biflorus</u> (horse gram)	Seed	Jaffé <u>et al.</u> (1973)
<u>Echinochloa frunetacea</u> (sanwa millet)	Seed	Kutty (1987)
<u>Eleusine coracana</u> (ragi)	Seed	Shivaraj and Pattabiraman (1980)
<u>Hordeum chilense</u> (barley)	Seed	Sanchez-Monge <u>et al.</u> (1987)
<u>Hordeum vulgare</u> (barley)	Seed	Mundy <u>et al.</u> (1983)
<u>Lens Culimaris</u> (lentils)	Seed	Jaffé <u>et al.</u> (1973)
<u>Mangifera indica</u> (mango)	Fruit	Mattoo and Modi (1970)
<u>Musa paradisiaca</u> (banana)	Fruit	Mattoo and Modi (1970)
<u>Panicum miliaceum</u> (proso)	Seed	Nagaraj and Pattabiraman (1985)
<u>Pennisetum typhodeum</u> (pearl millet)	Seed	Chandrasekher and Pattabiraman (1983)
<u>Phaseolus lunatus</u> (lima bean)	Seed	Jaffé <u>et al.</u> (1973)
<u>Phaseolus vulgaris</u> (cranberry bean)	Seed	Kotaru <u>et al.</u> (1987)
<u>Phaseolus vulgaris</u> (white kidney bean)	Seed	Yamaguchi (1991)
<u>Phaseolus vulgaris</u> (red kidney bean)	Seed	Powers and Whitaker (1977)
<u>Pisum sativum</u> (garden pea)	Seed	Jaffé <u>et al.</u> (1973)
<u>Secale cereale</u> (rye)	Seed	Granum (1978)
<u>Setaria italica</u> (setaria)	Seed	Chandrasekher <u>et al.</u> (1981)
<u>Sorghum bicolor</u> (sorghum)	Seed	Chandrasekher <u>et al.</u> (1981)
<u>Triticum aestivum</u> (wheat)	Seed	Bunocore <u>et al.</u> (1977)
<u>Zea mays</u> (maize)	Seed	Blanco-Labra and Iturbe-Chinas (1981)

1973 a,b; Koide et al., 1973). Further studies concerning its three-dimensional structure (Sweet et al., 1974) and its mechanism of interaction with its target enzyme (Baillargeon et al., 1980) made STI the first plant inhibitor to be well characterized. The soybean trypsin inhibitor (single chain) has a molecular weight of about 21 kDa (180 amino acids) and two disulphide bridges. STI is a single headed inhibitor of trypsin, but also inhibits chymotrypsin weakly at two reactive sites. The reactive site of STI is Arg(66)-Ile(64).

The complete primary structures of other legume seed proteins with sequence similarity to the Kunitz family has been determined. They are a basic trypsin inhibitor from winged bean (Psophocarpus tetragonolobus) (Yamamoto et al., 1983), a trypsin inhibitor from Erythrina latissima (Joubert et al., 1985), a trypsin inhibitor from the Brazilian Carolina tree (Adenanthera pavonina) (Richardson et al., 1986), an acidic trypsin inhibitor from P. tetragonolobus (Caldwell et al., 1990) and the major trypsin inhibitor (TICM6) from seeds of Prosopis juliflora (Negreiros et al., 1991). Lin et al. (1991) have purified a trypsin inhibitor (ACTI) from the seeds of Acacia confusa. Judging from its molecular weight (about 21 000), low half-cystine content and its amino-terminal sequence this inhibitor was considered to be a Kunitz-type inhibitor. The inhibitors from A. pavonina, P. juliflora and A. confusa all belonging to the subfamily Mimosideae are composed of two polypeptide chains (a large chain and a small one). There are suggestions that the two chains result from the proteolytic cleavage of a susceptible bond in a single-chain precursor

(Richardson, 1991; Negreiros et al., 1991).

Bifunctional amylase/proteinase inhibitors from cereal seeds are indeed homologous with the legume Kunitz inhibitors. They are isoform mixtures active against subtilisin and endogenous  $\alpha$ -amylases. These inhibitors are the barley amylase/subtilisin inhibitor (BASI) and the wheat amylase/subtilisin inhibitor (WASI) (see section 1.1.1). Also a rice amylase/subtilisin inhibitor has been identified belonging to the Kunitz family (Ohtsubo and Richardson, 1992). The cereal inhibitors are significantly closer to those from the Mimosideae than to those from the Leguminosae sub-families. The inhibitors from wheat (WASI) and barley (BASI), have Gly-Ala and Val-Ala respectively in the position homologous to the reactive site bond in the soybean protein. A X-ray crystallographic study of WASI is in progress (Maeda et al., 1987) and the identification of its reactive bond with subtilisin and of its contact areas with both subtilisin and endogenous  $\alpha$ -amylase will be very interesting.

#### **BOWMAN BIRK INHIBITOR FAMILY (BBI FAMILY)**

The trypsin inhibitor isolated by Bowman (1946) from soybean (Glycine max) had its characterization carried out by Birk et al. (1963a,b), hence its designation as Bowman-Birk inhibitor (BBI). This inhibitor (Odani and Ikenaka, 1972) together with a proteinase inhibitor from lima beans Phaseolus

lunatus (Tan and Stevens, 1971a,b) were the first enzyme inhibitors from plants to have their complete amino acid sequences determined. BBI is a protein of 71 amino acid residues with 7 disulphide bridges, that inhibits simultaneously, trypsin and chymotrypsin. The double-headed nature of this inhibitor was demonstrated by Odani and Ikenaka (1973), who cleaved the molecule by chemical (with cyanogen bromide) and enzymatic (using pepsin) methods into the two active domains without loss of inhibitory activity. The Bowman-Birk inhibitor has as reactive sites: lysine and serine (Lys-Ser) for trypsin and leucine and serine (Leu-Ser) for chymotrypsin.

Inhibitors of this type have been described in the seeds of many Leguminosae: garden bean Phaseolus vulgaris (Wilson and Lashowski, 1975), azuki bean Vigna angularis (Yoshikawa et al., 1979), mung bean Vigna radiata (Zhang et al., 1982; Wilson and Chen, 1983), cowpea Vigna unguiculata (Morhy and Ventura, 1987), Macrotyloma axillare (Joubert et al., 1979), peanut Arachis hypogaea (Norioka and Ikenaka, 1983) and faba bean Vicia faba (Asao et al., 1991).

It is now known that proteins with sequence similarity to the Bowman-Birk family are also found in cereals, wheat kernel (Poerio et al., 1989), rice bran (Tashiro et al., 1987), barley rootlets (Nagasue et al., 1988), seeds of foxtail millet (Tashiro et al., 1990) and in maize seedlings (Eckelkamp et al., 1993). Odani et al. (1986) have isolated two classes of inhibitors in wheat germ. One ( $M_r$  14 500) is a typical double-

headed and a second ( $M_r$  7 000) is a single-headed inhibitor.

An ancestral monovalent inhibitor that would have generated the double-headed ones by internal gene duplication has been postulated (Odani and Ikenaka, 1976). In this context, the more recent finding of single-headed inhibitors in wheat germ further supports the hypothesis and suggests that these are the most primitive among the known members of this family. Most of the members of the family exhibit numerous isoforms. Whilst these multiple forms may be produced by partial proteolysis during seed development (Madden *et al.*, 1985), many appear to be products of different genes (Tan-Wilson and Wilson, 1986). Molecular cloning of their genes and further proteins sequences will clarify more these evolutionary relationships.

#### POTATO I INHIBITOR FAMILY

The first member of this family was isolated from potato tubers and called the potato inhibitor I (Melville and Ryan, 1972). It is a strong inhibitor of chymotrypsin and also inhibits subtilisin. This inhibitor is an oligomer of  $M_r$  41 000 made of protomers of  $M_r$  8 100 that exist in at least ten isoelectric forms (Richardson *et al.*, 1976 a,b). The complete amino acid sequences of these protomers have been determined (Richardson, 1974; Richardson and Cossins, 1974, 1975). They contain only one disulphide bridge and the reactive site has been identified as Met/Leu (47)-Asp(48) (Richardson *et al.*,

1977).

This family is widespread since members are present in various plant families and also in an animal (the leech, Hirudo medicinalis) (Seemuller et al., 1980) and yeast (Maier et al., 1979). Plant species include members of the Solanaceae (potato and tomato, Melville and Ryan, 1972), Leguminosae (broad bean, Vicia faba, Svendsen et al., 1984), Gramineae (barley, Svendsen et al., 1982), Polygonaceae (buckwheat, Kiyohara and Iwasaki, 1985) and Cucurbitaceae (Cucurbita maxima, Krishnamoorthi et al., 1990).

Two chymotryptic inhibitors (CI-1 and CI-2) of this type are present in barley seeds, which also inhibit subtilisin. The amino acid sequence (deduced from cDNA) of a serine proteinase inhibitor ( $M_r$  8 100, named inhibitor I) induced in the leaves of tomato by wounding is 71% homologous with the potato inhibitor I (Kuo et al., 1984; Graham et al., 1985). The homologies found between the tomato inhibitor and those from barley CI-1, barley CI-2 and broad bean are respectively 25%, 33% and 35% (Garcia-Olmedo et al., 1987). All of the plant inhibitors in this family show a strong homology (approx. 36%) with the leech inhibitor (Richardson, 1991), whereas the homology with the yeast inhibitor is weak (approx. 16%) (Garcia-Olmedo et al., 1987).

The barley CI-2 and leech inhibitors are unusual because they do not contain disulphide bonds. Their three dimensional structure were determined by X-ray crystallography in order to understand which features contribute to their stability (McPhalen and James, 1987; McPhalen et al., 1985;

Bode et al., 1986).

#### POTATO II INHIBITOR FAMILY

The first protein of the potato II family to have its complete primary structure deduced was the small trypsin inhibitor from aubergine (Solanum melongena). Its reactive site was identified as the peptide bond Arg (38)-Asn(39) (Richardson et al., 1979). Besides the potato I inhibitor another inhibitor has been shown to be present in potato tubers, the named potato II inhibitor. Also a larger ( $M_r$  12 300) inhibitor II was subsequently demonstrated in wounded leaves of tomato (Plunkett et al., 1982). The complete amino acid sequences of inhibitors II from potato and tomato have been deduced from the corresponding cDNA clones (Sanchez-Serrano et al., 1986). These inhibitors showed a high degree of homology (54-84%). Other members of this family are a polypeptide chymotrypsin inhibitor (PCI) and a polypeptide trypsin inhibitor (PTI) isolated from potato tubers (Hass et al., 1982).

#### CUCURBIT (SQUASH) FAMILY

The discovery of this family is more recent. The members are all inhibitors of trypsin and the Hageman factor, and have been isolated from the seeds of the family Cucurbitaceae (Polanowski et al., 1980; Hojima et al., 1980, 1982; Leluk et al., 1983; Nishino et al., 1992). They are the smallest known plant inhibitors of serine proteinases, all

contain about 29 amino acids. These inhibitors are cross linked by three disulphide bridges and contain Arg-Ile or Lys-Ile at the reactive site (Wilusz et al., 1983; Joubert, 1984; Wieczorek et al., 1985). They are very strong inhibitors. The isooinhibitor forms reported seem to result from multiple structural genes and from proteolytic processing. Up to now these inhibitors have been found only within the Cucurbitaceae family. It will be of interest to investigate their range of distribution and variation.

#### CEREAL INHIBITOR SUPERFAMILY

The cereal superfamily includes inhibitors of  $\alpha$ -amylases, inhibitors of proteinases, bifunctional inhibitors and proteins with no known inhibitory activity, grouped together on the basis of sequence homology.

This family is the one where many of plant  $\alpha$ -amylase inhibitors are found. Some have their sequence determined: wheat (two inhibitors, a monomeric 13 kDa form and another dimeric 26 kDa form) (Kashlan and Richardson, 1981; Maeda et al., 1985) and rye (N-terminal sequence), by Lyons et al., 1987. Primary structures of other inhibitors such as a bifunctional amylase/trypsin inhibitor (ragi I-1) from ragi Eleusine coracana (Campos and Richardson, 1983) and trypsin inhibitors from barley (Odani et al., 1983a) and maize (Mahoney et al., 1984) exhibit high levels of homology (50-76%) between them and with the  $\alpha$ -amylase inhibitors (24-26%).

Barley, wheat and rice contain proteins which can be

extracted by chloroform/methanol, the so-called CM-proteins (Garcia-Olmedo et al., 1982). N-terminal and complete amino acid sequences of these proteins show homology with the cereal proteinase and  $\alpha$ -amylase inhibitors. The roles of the CM-proteins are unknown but homology with the enzyme inhibitors indicates a probable function. CMa was also shown to be an inhibitor of  $\alpha$ -amylase from the insect Tenebrio molitor, while CMc and CMd inhibit trypsin (Barber et al., 1986).

This family also has low homology with the 2S storage proteins from castor bean (Odani et al., 1983b), oil-seed rape (Crouch et al., 1983) and Brazil nut (Ampe et al., 1986).

#### **RAGI I-2 FAMILY**

Shivaraj and Pattabiraman (1980) have isolated another  $\alpha$ -amylase inhibitor (ragi I-2) from seeds of ragi Eleusine coracana. Its complete amino acid sequence was determined by Campos and Richardson (1984) who found no homology with the bifunctional  $\alpha$ -amylase/trypsin inhibitor (ragi I-1) isolated from the same seed or with any other  $\alpha$ -amylase inhibitor. This fact led these researchers to suggest a new family. The sequence contains two regions of internal homology indicating the occurrence of gene duplication. A related protein (a probable  $\alpha$ -amylase/proteinase inhibitor) from barley aleurone layers had its sequence deduced from the corresponding cDNA and showed 50% homology with the ragi I-2 amylase inhibitor. Another protein with no known inhibitory

activity isolated by Yu et al. (1988) from rice seeds has 76% and 63% homology with the ragi I-2 inhibitor and barley protein respectively.

#### MAIZE 22 kDa/THAUMATIN/PR FAMILY

Richardson et al. (1987) purified and sequenced a bifunctional  $\alpha$ -amylase/trypsin inhibitor from maize. This contained 206 amino acids ( $M_r$  22 077) with high contents of Ala, Gly and Cys. Surprising results were found when the maize inhibitor sequence was compared to a protein databank. It showed homology with three distinct proteins: a sweet protein (thaumatin) from the fruits of Thaumatococcus danielli (Edens et al., 1982), a PR ("pathogenesis related") protein induced in tobacco plants by virus infection (Cornelissen et al., 1986) a 26 kDa protein (osmotin) which accumulates in cultured cells of tobacco adapted to grow under osmotic stress (Singh et al., 1987). Richardson (1991) suggests that this sequence similarity indicated a role for thaumatin, the tobacco PR protein and the maize inhibitor as inhibitors of hydrolytic enzymes. However other workers were unable to detect inhibitory activity when the PR protein was tested against trypsin (Pierpoint et al., 1987).

#### CYSTATIN FAMILY

Cystatins are a superfamily of cysteine proteinase inhibitors (Barrett et al., 1986). They are reversible

competitive inhibitors with high affinities for their target enzymes. Although most cystatins have been isolated from animal sources, a strong inhibitor (called oryzacystatin) of cysteine proteinase was isolated from rice seeds (Abe et al., 1987). Its primary structure shows significant homology (30%) with the human cystatin A and with many other animal cystatins (e.g. plasma and chicken egg white (Abe et al., 1987). A similar inhibitor has also been found in seeds of the legume Wisteria floribunda (Hirashiki et al., 1990).

### 1.1.3 Biological Roles of Plant Enzyme Inhibitors

As yet the exact biological role of plant enzyme inhibitors is unknown but there are indications of some functions. Possible roles are that they act as storage proteins, as regulators of endogenous enzymes, or as defensive agents against the attack of insects or pathogens.

#### STORAGE PROTEINS

Several features (resistance to denaturation by heat, extremes of pH and many proteolytic enzymes) suggest that plant enzyme inhibitors may act as storage proteins (Richardson, 1991). The large quantities of proteinase inhibitors present in some seeds and tubers is also suggestive of a role as storage proteins. They represent about 6% of the protein in soybean (Rackis and Anderson, 1964), up to 10% of the total protein of barley seeds (Mikola and Kirsi, 1972), over 10% of

the soluble proteins in potato tubers (Weder, 1981) and about 20% of the soluble proteins of seeds of the Brazilian Carolina tree Adenanthera pavonina (Richardson et al., 1986).

During germination of some seeds a decrease of inhibitor activity was observed. This fact, together with the high levels of inhibitors mentioned above suggests a secondary storage role instead a primary metabolic one. Pace et al. (1978) noted that the amount of wheat  $\alpha$ -amylase inhibitors decreased rapidly on germination. However, the levels of some proteinase inhibitors have been found to increase with germination (Pusztai, 1972). On the other hand, Carasco and Xavier-Filho (1981) found that biosynthesis of inhibitors occurs at the same time as that of the reserve proteins in developing seeds of cowpea (Vigna unguiculata). There are also reports of similar events in developing seeds of other species (Kute et al., 1984; Mundy and Rogers, 1986; Buonocore and Silano, 1986).

#### **REGULATORS OF ENDOGENOUS ENZYMES**

The fact that trypsin-like enzymes or other serine class proteinases were not commonly detected in plants did not support a role in the control of proteolysis for the plant proteinase inhibitors (Xavier Filho, 1992). Only a few inhibitors are known which inhibit the endogenous trypsin-like proteinases of seeds (Richardson, 1991). Also it appears that cysteine proteinases are apparently more important than serine proeinases in the mobilization of proteins during germination

of seeds (Huffaker, 1990). Control of the activity of these enzymes is certainly performed through the action of the cysteine proteinase inhibitors which are found in seeds (Xavier Filho, 1992).

The presence of inhibitors of endogenous  $\alpha$ -amylases in cereals, e.g. wheat (Mundy et al., 1984); barley (Hejgaard et al., 1983) and rye (Weselake et al., 1985), is further evidence that these inhibitors may act as regulators of endogenous  $\alpha$ -amylase activity, for example in starch metabolism during seed development and germination.

#### DEFENSE AGAINST INSECTS AND PATHOGENS

The effects of enzyme inhibitors in plant defense mechanisms have been investigated after the initial observations by Michel and Standish (1947) that larvae of Tribolium castaneum were unable to develop normally on soybean seeds. Since then, a large body of information has been obtained on the possible involvement of enzyme inhibitors in plant defense mechanisms (Ryan, 1973; Richardson, 1981; Boisen, 1983; Richardson, 1991; Ryan, 1990; Xavier Filho, 1992).

Initially it was thought that trypsin-like serine proteinases were the major gut digestive enzymes of insects. Further studies in vitro and in vivo have been carried out on the digestive enzymes present in insects guts as well as their interaction with plant enzyme inhibitors. Serine proteinases have been identified in extracts from the digestive tracts of insects from many families, particularly those of the

Lepidoptera, and many of these enzymes are inhibited by proteinase inhibitors. In these insects, the pH optima of the guts are in the range of 9-11, where serine proteinases are most active (Applebaum, 1985). In 1979 Gatehouse et al., suggested that the resistance of a cowpea (Vigna unguiculata) cultivar (TVu2027) towards the bruchid Callosobruchus maculatus was due to the unusually high level of trypsin inhibitors present in the seeds. Furthermore, Gatehouse and Boulter (1983) showed that cowpea trypsin inhibitors at 10% of the diet were toxic to larvae of the C. maculatus. This antimetabolic response could be reversed by the addition of methionine or cysteine to the diets. The authors suggested that ingestion of inhibitors alone does not cause the adverse effects, but causes hyperproduction of trypsin which may enhance the loss of sulfur amino acids. On the other hand, Xavier-Filho et al. (1987) examined the levels of inhibitors of different proteinases (trypsin, chymotrypsin, subtilisin and papain) in susceptible (CE 11, CE 31 and CE 524) and resistant (TVu2027, IT8ID 1064 and IT8ID 1045) cowpea V. unguiculata cultivars and found no correlation between the levels of inhibitors and resistance to attack by C. maculatus. It is interesting to note the observations by Roy and Bhat (1975) that two out of ten varieties of Lathyrus sativus seeds with lower levels of trypsin inhibitors were somewhat protected from infestation by Callosobruchus chinensis.

Direct evidence of the role of the cowpea trypsin inhibitor in plant defense mechanism was first obtained by Hilder et al. (1987) when they showed that tobacco plants

transformed with a gene encoding the cowpea inhibitor were more resistant to feeding by larvae of Heliothis virescens, a lepidopterous pest, than untransformed control plants. Johnson et al. (1989) transformed tobacco plants with genes coding for tomato and potato inhibitor II proteins (having chymotrypsin and trypsin inhibitory activities). Leaves of plants expressing the inhibitor II proteins at levels of 50 µg/g tissue, when supplied to larvae of Manduca sexta caused severe inhibition of growth as compared to larvae feeding on untransformed plants. At higher levels (100 µg/g tissue) of inhibitors the larvae grew even less and some died.

Three proteinases and four α-amylases were identified in C. maculatus larvae. All proteinases had the same  $M_r$  (25 000) and optimum pH (between 5.5 and 6.0) and were inhibited by protein inhibitors of cysteine proteinases isolated from V. unguiculata seeds. The four α-amylases had  $M_r$  of 56 000, 45 000, 36 000 and 33 000, and none were inhibited by an α-amylase inhibitor from Phaseolus vulgaris seeds. In contrast an α-amylase inhibitor from wheat inhibited the amylases of  $M_r$  45 000, 36 000 and 33 000 but failed to inhibit that of  $M_r$  56 000 (Campos et al., 1989). Earlier observations indicated the presence in C. maculatus larvae of proteinases with maximum activity at pH 5.5 (Xavier Filho and Coelho, 1980), while Gatehouse et al. (1985) and Kitch and Murdock (1986) who suggested that the major proteinase present in the guts of C. maculatus belonged to the cysteine proteinases family. Similar cysteine proteinases have now been isolated from midguts of the flour beetle Tribolium castaneum, and the

Mexican beetle Epilachna varivestis (Murdock et al., 1988). Gatehouse et al. (1986) who tested wheat  $\alpha$ -amylase inhibitors with  $\alpha$ -amylases from Tribolium confusum (a storage pest of wheat) and Callosobruchus maculatus (a storage pest of legumes), and showed that the inhibitors inhibited both insect amylases in vitro. However, the wheat  $\alpha$ -amylase inhibitors inhibited only the C. maculatus in vivo. Amylases extracted from several insects such as the mediterranean flour moth larvae (Anagasta kunhiella), red flour beetle adults (Tribolium castaneum), adults and larvae of confused flour beetle (Tribolium confusum) were inhibited by a red kidney bean amylase inhibitor (Powers and Culbertson, 1982).  $\alpha$ -Amylases in homogenates of midguts of larvae of the granary weevil (Sitophilus granarius) and maize weevil (Sitophilus zeamais) were also studied, showing inhibition by  $\text{NaHCO}_3$  extracts of wheat (Baker, 1983). More recently, Chen et al. (1992) isolated two  $\alpha$ -amylases from adults of both the rice weevil, Sitophilus oryzae and the red flour beetle Tribolium castaneum and a single  $\alpha$ -amylase from adults of the yellow mealworm, Tenebrio molitor. All of the purified enzymes were inhibited by an inhibitor from wheat, whereas an inhibitor from corn inhibited only the enzymes from the red flour beetle and yellow mealworm.

Plant enzyme inhibitors may play an active role in the defense of the plant against herbivorous insects, not only as constitutive but also as induced defenses. The participation of proteinase inhibitors in induced defenses of plants was first suggested by Green and Ryan (1972) who demonstrated that mechanical wounding or insect damage to leaves of potato or

tomato led to a local accumulation of proteinase inhibitors. Later it was shown (Ryan, 1973) that this "primitive immune response" is systemic and regulated by a factor named PIIF (proteinase inhibitor inducing factor). In feeding studies with Spodoptera exigua, a pest of many agricultural crops including soybean, tomato and potato, it was shown that leaves from tomato plants wounded by insects were a poorer food source than from unwounded plants (Ryan et al., 1986). Peng and Black (1976) found that levels of trypsin inhibitor increased more in leaves in varieties of tomato that were resistant to Phytophthora infestans than in susceptible varieties. Broadway et al. (1986) demonstrated that leaves from wounded tomato plants accumulate potato inhibitors I and II in the leaves resulting in reduced growth of S.exigua. Similarly, a trypsin inhibitor was shown to be wound induced in leaves of alfalfa (Brown and Ryan, 1984).

Evidence for a possible role of plant proteinase and  $\alpha$ -amylase inhibitors in defense against microbial plant pathogens is just emerging. Inhibitors that inhibit proteolytic enzymes from microorganisms are common in plants (mainly legumes and cereals), e.g. inhibitors of subtilisin, a serine protease secreted by Bacillus subtilis (Garcia-Olmedo et al., 1987). Other examples are a proteinase inhibitor from maize that is active against a fungal (Fusarium moliniforme) enzyme (Kolaczowska et al., 1980) and a thiol proteinase inhibitor present in some varieties of cowpea which are resistant to the cowpea mosaic virus (Bruening and Sanderson, 1984). Many

endogenous  $\alpha$ -amylase inhibitors from cereals are also active against bacterial or fungal enzymes (Silano, 1986), hence they may play a protective role by inhibiting microbial proteinases.

Since inhibitors of proteinases and  $\alpha$ -amylase inhibitors seem to have a role in natural plant defense, their genes have considerable potential for the improvement of the plant defense systems either through traditional breeding methods or through direct transfer of inhibitor genes via DNA technologies to target specific digestive enzymes of insects pests and pathogens. A good example is the cowpea trypsin inhibitor gene which has been shown to give some measure of field resistance when transferred to tobacco (Hilder *et al.*, 1987). The transfer to other crops important to third world countries of genes encoding plant proteins involved in defense, such as the trypsin inhibitor gene from cowpea, could be a more economically and environmentally sensible way of increasing agricultural productivity.

## 1.2 Plant Chitinases, $\beta$ -1,3-Glucanases and Lysozymes

Bowles (1990) has divided the plant defense-related proteins into three classes based on their role in the defense function. The first class includes proteins that change the properties of the plant extracellular matrix (e.g. glycine-rich proteins). A second class comprises proteins associated with deterrence and antimicrobial activity such as enzyme inhibitors (see section 1.1.3). Other proteins belonging to this class are hydrolases such as chitinases,  $\beta$ -1,3-glucanases and lysozymes.

These enzymes have a potential function in disease resistance which is supported by the finding that they are often induced in response to a pathogen attack (Boller, 1985). The third class includes proteins whose expression is induced as a response to damage or infection, but which are of unknown function. Some PR("pathogenesis related") proteins are included in this class, while others have been shown to have biological activity, for example as chitinases or glucanases. The biological roles of the PR proteins will be discussed, later (see section 1.3).

#### 1.2.1 Potential Function in Disease Resistance

Plant chitinase was first discovered in bean seeds (Powning and Irzykiewicz, 1965), and it has subsequently, been showed that endochitinases (chitinases with endo-activity) are widespread in higher plants. They have been reported in healthy tissues such as wheat seeds (Molano et al., 1979), tomato stem (Pegg and Young, 1982), barley seeds (Leah et al., 1987), soybean seeds (Wadsworth and Zikahis, 1984), oat leaves (Fink et al., 1988) and potato (Gaynor, 1988) and yam (Araki et al., 1992) tubers. Hart et al. (1992) reported the crystallization of the barley endochitinase (Leah et al., 1987) as a preliminary step in the investigation of its structure and action.  $\beta$ -1,3- Glucanase is also found in higher plants and has been purified from several sources, Nicotiana glutinosa (Moore and Stone, 1972), Pisum sativum (Wong and Machlachlan, 1979), tomato (Young and Pegg. 1981) tobacco (Felix and Meins,

1985) and cell suspension cultures of barley (Hordeum vulgare) (Kragh et al., 1991). More recently a  $\beta$ -1,3-glucanase and two chitinases were purified from chickpea cell-suspension cultures (Vogelsang and Barz, 1993). Lysozyme was discovered by Fleming (1922) in animal tissues and secretions. Later lysozyme was purified from the latex of certain plants; papaya (Carica papaya, Howard and Glazer, 1969), fig (Ficus, Glazer et al., 1969) and Hevea brasiliensis (the purified enzyme was named "hevamine", Tata et al., 1983). In each case the purified enzyme also exhibited a high chitinase activity. The lysozymes from turnip (Bernier et al., 1971) and from a Rubus cell culture (Bernasconi et al., 1985) were also purified and shown to act as endochitinases. More recently it has been reported (Majeau et al., 1990) that cucumber seeds contain a highly basic endochitinase with lysozyme activity. Jekel et al. (1991) determined the primary structure of hevamine, the enzyme with lysozyme/chitinase activity from Hevea brasiliensis latex. The sequence has about 60% identity with that of a chitinase from cucumber (Metraux et al., 1989) and 95% with the N-terminal sequence of the lysozyme/chitinase of Parthenocissus quinquefolia (Bernasconi et al., 1987).

Chitinase has often been regarded in plants as a "secondary enzyme" with regard to the primary metabolism, since its substrate, chitin (a linear polymer of  $\beta$  (1  $\rightarrow$  4)N-acetylglucosamine), does not occur in higher plants. However, chitin is present in the cell walls of many fungi, and chitinase may therefore be primary with regard to plant disease resistance (Boller et al., 1983). On the other hand,

chitinases and  $\beta$ -glucanases are not only produced constitutively in some tissues but may also be induced by infection with pathogens, by treatment with the plant hormone ethylene, or by elicitors (e.g. cell wall components of pathogenic fungi) (Boller, 1985). This co-occurrence and co-regulation in some situations indicates a common role for the two enzymes in plant defense (Pegg and Young, 1981; Mauch et al., 1984). Despite the fact that lysozyme is considered an important defense mechanism of animals against bacterial infection (Jollès and Jollès, 1984), there are reports that it can also have a function in plant defense. A summary of these findings is presented here.

Wargo (1975) showed that partially purified chitinase and  $\beta$ -1,3-glucanase from stem and root tissues of sugar maple (Acer saccharum), red oak (Quercus rubra), black oak (Q. velutina) and white oak (Q. alba) lysed the hyphal walls of Armillaria mellea. Lysis of fungi was observed in the mycorrhizal symbiosis of orchids with fungi, and plant chitinase and  $\beta$ -1,3-glucanase were implicated in this process (Pegg, 1977). Inhibition of fungal growth by wheat germ agglutinin preparations, a property previously attributed to lectin activity (Mirelman et al., 1975), was in fact due to the presence of chitinase as a contaminant (Molano et al., 1979). In addition to this discovery, Schlumbaum et al. (1986) showed that plant chitinases but not chitin-binding lectins, are potent antifungal proteins in plants. Infection of the leaves of cucumber plants with tobacco necrosis virus (TMV) or with Colletotrichum lagenarium or other fungal pathogens caused a

strong increase in chitinase activity in the infected leaf (Métraux and Boller, 1986). Mercer et al. (1992) reported that chitinase from Phaseolus vulgaris caused premature hatch of nematode eggs and could be used as an aid in the control of nematodes. Daugrois et al. (1992) purified and characterized two basic  $\beta$ -1,3-glucanases induced in bean seedlings by infection with Colletotrichum lindemuthianum. Infection of potato leaves with the fungal pathogen Phytophthora infestans caused a similar, strong and co-ordinated induction of  $\beta$ -1,3-glucanases and chitinases (Schroder et al., 1992). Chitinase activities have been reported in Allium and Pisum roots colonized by Glomus species (Dumas-Gaudot et al., 1992). The induction of chitinase was also stimulated by the addition of mycelial walls of the fungus Chaetomium globosum to cultured carrot cells (Kurosaki et al., 1986). Roby and Esquerre-Tugaye (1987) isolated six different chitinases from melon plants infected with the fungal pathogen Colletotrichum lagenarium, and showed that the major enzymes, chitinase I and chitinase II, acted as an endochitinase and as an exochitinase respectively.  $\beta$ -1,3-Glucanase from soybean was shown to release elicitors of phytoalexins (secondary metabolites that are toxic to bacteria and fungi) from isolated fungal cell wall (Keen and Yoshikawa, 1983). An endochitinase was induced by ethylene in primary leaves of Phaseolus vulgaris (Boller et al., 1983). The purified enzyme attacked chitin in isolated cell walls of the fungus Fusarium solani, and also acted as a lysozyme when incubated with the bacterium Micrococcus lysodeikticus. Hirano and Nagoo (1989) showed that lysozyme

inhibits the growth of several phytopathogens. In pea pods, elicitors and fungal infections caused enhanced production of ethylene; but only later were chitinase and  $\beta$ -1,3-glucanase induced. When this production of ethylene was blocked by aminoethoxyvinylglycine, the induction of chitinase and  $\beta$ -1,3-glucanase remained unaffected. This indicates that the ethylene produced was not the signal for the induction of the two enzymes (Mauch et al., 1984). Chitinase and  $\beta$ -1,3-glucanase induction in Phaseolus vulgaris by cell wall elicitor from Colletotrichum lindemuthianum has been studied together with the effects of the hormones IAA and ethylene (Hughes and Dickerson, 1991). Chitinase and  $\beta$ -1,3-glucanase increased in response to the elicitor in the resistant cultivar, Kievit, but not in the susceptible cultivar, Pinto. However, both activities increased in both cultivars in response to hormones. It is of interest that a recent study (Leung, 1992) reported the involvement of plant chitinase in sexual reproduction of higher plants.

#### 1.2.1.1 Regulation of Chitinase Gene Expression

Several studies of the molecular mechanisms controlling chitinase gene expression have been reported. Broglie et al. (1986) presented evidence that treatment of bean seedlings with ethylene resulted in an increase in steady-state chitinase mRNA levels, indicating that the control of chitinase gene expression by ethylene occurs at the level of

gene transcription. In vitro translation of mRNAs from healthy melon plants and plants infected with Colletotrichum lagenarium, demonstrated the presence of chitinases mRNA in infected but not in healthy melon seedlings. From these results it was concluded that infection of melon seedlings by a pathogen caused an increase in the translatable mRNAs for host chitinases (Roby and Esquerre-Tugaye, 1987). Vögeli et al. (1988) also showed that the levels of chitinase and  $\beta$ -1,3-glucanase mRNAs in bean leaves were regulated by ethylene. A cDNA clone encoding an endochitinase was isolated from potato leaves. The expression of hybridising mRNAs was tightly regulated by the phytohormone ethylene, showing an induction of approximately 30-fold in young leaves. It has also been shown that this gene is expressed in leaves, roots, stems and petioles of the mature potato plant (Gaynor, 1988). Simmons et al. (1992) sequenced a rice  $\beta$ -glucanase gene and its expression was analysed at the level of mRNA accumulation. This gene was expressed at higher levels when treated with ethylene, fungal elicitors and in response to wounding. Jutidamrongphan et al. (1991) reported the sequence of a partial cDNA clone corresponding to a mRNA induced in leaves of barley by infection with fungal pathogens which matched almost perfectly with of a cDNA clone coding for  $\beta$ -1,3-glucanase isolated from the scutellum of barley. These authors suggest that activation of  $\beta$ -1,3-glucanase genes may be a general response of cereals to infection by fungal pathogens.

### 1.2.1.2 Localization

In general, plant hydrolases such as chitinases and  $\beta$ -1,3-glucanases are located in the "lytic compartment" of the cells, which includes the vacuole and the cell wall space (Matile, 1984). A cell wall location appears to be well-suited for a function in defense, since phytopathogens attack plant cells from outside and often have to penetrate the host cell wall. Interestingly, a chitinase induced by ethylene in bean leaves was found to be located only in the vacuoles (Boller and Vögeli, 1984). Many other compounds which are important in defense are also localized in the vacuoles (Matile, 1984). However, experiments reported by Kurosaki *et al.* (1986) with chitinase secreted from cultured carrot cells treated with fungal mycelial walls suggested that the induced chitinase was secreted into the apoplastic space of the cells. The localization of systemically induced chitinase activity has been examined in the second leaves of cucumber plants that have been infected on the first leaf with tobacco necrosis virus. The data indicated that most of the chitinase activity was located in the extracellular space (Boller and Metraux, 1988). Mauch and Staehelin (1989), based on their studies of the subcellular localization of ethylene-induced chitinase and  $\beta$ -1,3-glucanase in bean leaves, suggested some functional implications in plant defense for both enzymes. They found that chitinase and  $\beta$ -1,3-glucanase were accumulated in the vacuole of ethylene-treated leaf cells, while a second but minor site of accumulation of  $\beta$ -1,3-glucanase was the cell wall. By

carrying out fractionation studies they confirmed the accumulation of high concentrations of both chitinase and  $\beta$ -1,3-glucanase in isolated vacuoles, and demonstrated that only  $\beta$ -1,3-glucanase, but not chitinase, was present in intercellular fluids collected from ethylene-treated leaves. They therefore proposed that the vacuole-localized chitinase and  $\beta$ -1,3-glucanase constitute a last line of defense to be released when the host cells are lysed. On the other hand, they suggested that  $\beta$ -1,3-glucanases localized in the cell wall could be involved in recognition processes. Mauch et al. (1992) studied the spatial pattern of accumulation of chitinase and  $\beta$ -1,3-glucanase in ethylene-treated leaves of bean (Phaseolus vulgaris). Their results suggest that both activities accumulate specifically in the lower epidermis and along vascular strands of bean leaves.

More specific knowledge of plant vacuolar targeting sequences is now being gained. Evidence from the targeting of glucanases and chitinases to the vacuole in dicotyledonous plants suggests that the signal for targeting to the vacuole is at the C-terminal end of the molecule (Miflin and Shewry, 1991). For example, tobacco chitinases can be found in the vacuole and in the extracellular space of plant cells. A comparison of their deduced amino acid sequences indicate the presence of C-terminal extensions on the vacuolar forms. The vacuolar form of chitinase has Glu-Leu-Leu-Val-Asp-Thr-Met as its C-terminus, and this domain is absent from the homologous cell chitinase (Chrispeels and Raikhel, 1992).

### 1.3 PR ("Pathogenesis Related") Proteins

When some plants, including monocotyledons, are infected with viruses or other pathogens the development of symptoms is accompanied by the appearance of substantial amounts of proteins named PR ("pathogenesis-related") proteins, (Pierpoint et al., 1981; Pierpoint and Shewry, 1987). PR proteins were first identified in extracts from the leaves of tobacco (Nicotiana tabacum) cultivars which reacted hypersensitively to tobacco mosaic virus (TMV). After further characterization of these proteins some common properties became evident. Such proteins are generally of low molecular weight, acid-soluble and resistant to proteolysis (van Loon, 1985). They are localized predominantly in the intercellular spaces (Parent et al., 1984). In addition to the four well known tobacco (cv. Xanthi-nc) PR proteins (PR-Ia, PR-Ib, PR-Ic and PR-II), Pierpoint (1986) distinguished other PR proteins by electrophoresis and chromatofocusing. These are referred to as N, O, P, Q, R and O', P', Q' and R', the last four proteins having electrophoretic mobilities similar to O, P, Q and R. By using high-performance chromatography Kauffman et al. (1990) purified and characterized four new PR proteins from tobacco (N. tabacum c.v. Samsun NN) leaves infected with TMV. They were designated as PR-s<sub>1</sub>, PR-s<sub>2</sub>, PR-r<sub>1</sub> and PR-r<sub>2</sub>. When immunoblotting experiments were carried out, no cross-reaction was detected between an antiserum raised against PR proteins R and S and the four new tobacco PR proteins r<sub>1</sub>, r<sub>2</sub>, s<sub>1</sub> and s<sub>2</sub>. Although no conclusive role can yet be assigned to these

proteins, some proposed functions will be described here.

Since PR proteins can also be induced by the application of a variety of chemicals (e.g. acetyl salicylic acid( aspirin) and ethylene), by osmotic stress, by flowering and by plasmolysis it was proposed that induction might be result of some type of stress (Pierpoint, 1986; Wagih and Coutts, 1981).

PR proteins are present in uninfected tissue only at very low concentrations (Antoniw et al., 1985). They appeared in large amounts in hypersensitively responding plants, both in inoculated leaves and in neighbouring uninoculated, virus free leaves (Carr et al., 1982). Leaves in which PR proteins were induced by ethylene or aspirin produced fewer and smaller lesions on subsequent inoculation with tobacco mosaic virus (van Loon, 1985). Thus, PR proteins are associated with "systemic acquired resistance", the phenomenon of increased resistance to infection that occurs after initial infections. Similar systemic production of protein enzyme inhibitors occurs following the mechanical injury of many plants, and may confer some protection against predation by insects (Ryan, 1973). Determination of the complete amino acid sequence (Richardson et al., 1987) of a trypsin/ amylase inhibitor of maize showed not only a high level of sequence identity (52%) with a sweet protein thaumatin, but also similar sequence identity (57%) with a PR protein induced in tobacco plants following infection with TMV. These reports suggest that this group of PR proteins should be assayed for their activity against hydrolytic

enzymes.

Legrand et al. (1987) purified and characterized four endochitinases from leaves of tobacco (cv. Samsun NN) reacting hypersensitively to tobacco mosaic virus. Two of them were acidic chitinases and were identified as the tobacco PR proteins named PR-P and PR-Q. Payne et al. (1990) have also described the isolation and characterization of cDNA clones encoding two acidic isoforms of tobacco chitinase which correspond to PR-P and PR-Q. Eight PR proteins were identified in maize leaves after mercuric chloride treatment or infection with brome mosaic virus. They were called PRm (pathogenesis-related maize) proteins and four were found to be chitinases (Nasser et al., 1988). Pierpoint et al. (1990) have studied eight intercellular proteins that appeared in leaves of potato plants after spraying with salicylate. They resembled the PR proteins of tobacco leaves and evidence from amino acid sequences and other studies indicated that three of them were a thaumatin-like protein, an acidic chitinase and a  $\beta$ -1,3-glucanase. Edelbaum et al. (1991) reported that two antiviral proteins, gp35 and gp22, correspond to  $\beta$ -1,3-glucanase and an isoform of PR-5. One chitinase corresponding to PR-P was enhanced locally and systemically after inoculation with TMV or treatment with ethephon (Ye et al., 1992). In addition to the reports indicating that some PR proteins have chitinase activity, we can also note certain common properties between these two groups of proteins. They are both induced by ethylene, by pathogen attack, by biotic and abiotic elicitors and systemically by local necrosis (Legrand et al., 1987).

Also, we can note that the production of PR proteins increases in the resistance of plants to viruses while a potential function of chitinases and  $\beta$ -1,3-glucanases in disease resistance of plants (see sections 1.2.1) has been observed.

Further information on the structures and relationships of PR proteins is required in order to evaluate their functions in diseased and stressed plants.

#### 1.4 Aims of the Project

Plant proteins are of considerable interest and importance, particularly in tropical and underdeveloped areas where animal protein is not easily available (Chaves et al., 1962). Seeds of cereal and legumes and their products make up a major part of human diet. Cereals account for about 45% of the daily per capita protein supply in the world and approximately 63% in developing countries. In view of the nutritional importance of cereal grains a lot of work has been carried out on the structure and molecular biology of their storage proteins (Lumen, 1990; Kreis et al., 1985).

Among plant proteins, the group of enzyme inhibitors, has received special attention because of their possible biological roles. These inhibitors can play a role in plant kingdom as reserve proteins, as regulators of endogenous enzymes and as defensive agents against insect pests and pathogens. They also have potential toxic effects in animal and human nutrition (Richardson, 1991).

The cereal Job's tears (Coix lachryma-jobi) is native

to India, Burma, China and Malaysia. A number of varieties exist that differ in plant size and seed structure. Some seeds with a tear-shaped hard shell are used throughout the Orient for ornamental purposes. In the tropics they are made into rosaries and sold to tourists. A thin-shelled type used as food is known as "adlay" in the Philippines and South America and as "ma-yuen" in China. The Chinese use it in soups in the same way as pearl barley is used. A drink is made from it in Japan, and it is sometimes made into beer. The whole plant can also be used for forage and fodder. The bran is used in folk medicine in Asia. Thin-shelled edible types of Coix were introduced into Brazil around 1939 and were subsequently subjected to breeding programs to improve agronomic characteristics such as yield and growth period (Vallaey's, 1948; von Schaaffhausen, 1952; Arora, 1977).

Some previous studies of Coix have been reported. Ohtsubo et al. (1985) purified and characterized a heat stable inhibitor (JBTI) from seeds of C.lachryma-jobi var. Ma-yuen. Changes in the levels and localization of proteases and JBTI were followed in developing seeds of Coix, and their interactions were examined (Ohtsubo et al., 1989). In addition a Brazilian research group characterized the storage proteins present in seeds of C. lachryma-jobi var. Adlay (Ottoboni et al., 1990). More recently, Zhe-fu et al. (1992) purified and characterized a 10kDa exochitinase from seeds of Coix lachryma-jobi.

The main objectives of this project were the purification, characterization and determination of the primary

structure of protein enzymes inhibitors, from seeds of C. lachryma-jobi. Particular attention was focused on  $\alpha$ -amylase inhibitors because no previous work appeared to have been done on proteins of this type from Coix. Also, it was thought that the determination of the primary structure of inhibitors might give important indications of the possible evolutionary relationships between these Coix proteins and other inhibitors.

Preliminary molecular studies were also carried out, including the isolation and in vitro translation of mRNA fractions from developing seeds.

CHAPTER 2

MATERIALS AND METHODS

## **MATERIALS**

### **2.1 Biological Materials**

#### **2.1.1 Origin of Seeds**

Seeds of Job's Tears (Coix lachryma-jobi L. var. Ma-yuen Stapf) were obtained from Dr. H. Nakano, Prefectural Agricultural Experimental Station, Okayama, Japan.

#### **2.1.2 Origin of Bean (Phaseolus vulgaris) chitinase cDNA.**

(Broglie et al., 1986)

The bean cDNA cloned into the EcoRI site of pUC13 (called pCh18) was kindly supplied by Dr. Jack Gaynor, The State University of New Jersey, Newark, New Jersey.

#### **2.1.3 Origin of Antisera to Sugar Beet Chitinase and Wheat Germ Chitinase**

The antisera for the sugar beet and wheat germ chitinases were obtained as a generous gift from Dr. Jorn Mikkelsen-DDS Copenhagen, Denmark.

## 2.1.4 Enzymes and other Biological Materials

### Materials

	<u>Manufacturer</u>
$\alpha$ -Amylase from <u>A. oryzae</u>	Sigma
$\alpha$ -Amylase from <u>B. subtilis</u>	Sigma
$\alpha$ -Amylase from Barley Malt (a mixture of $\alpha$ -amylase and $\beta$ -amylase)	Sigma
$\alpha$ -Amylase from Human Saliva	Sigma
$\alpha$ -Amylase from Porcine Pancreas	Sigma
Bovine Serum Albumin	BRL
$\alpha$ -Chymotrypsin	Worthington Bioc. Corp.
$\lambda$ - <u>HindIII</u> DNA Size Markers	BRL
Protein-A-Alkaline Phosphatase	Sigma
Restriction Endonucleases	Sigma
<u>S. aureus</u> V8 Protease	Worthington Bioc. Corp.
Trypsin TPCK-Treated	Worthington Bioc. Corp.
Trypsin Type XI from Porcine Pancreas	Sigma

## 2.2 Chemicals

	<u>Manufacturer</u>
Acetic acid (glacial) (AR grade)	Fisons
Acetonitrile (HPLC grade S)	Rathburn Chemicals Ltd.
Acrylamide (gel grade)	Sigma
Adenosine 2'.3' monophosphate 2'.3' AMP (mixed isomers)	Sigma
Agarose (medium EEO)	Sigma
Ammonium acetate (AR grade)	Fisons
Ammonium sulphate	BDH
Ammonium persulphate (electrophoresis grade)	Fisons
N-Benzoyl-DL-arginine-p- nitroanilide-HCl(BAPNA)	Sigma
Bio-gel P2	Bio-Rad
Bis acrylamide (electran grade)	BDH
5-bromo-4-chloro-3-indolyl phosphate	Sigma
Bromophenol blue	Sigma
Butan-1-ol	Sigma
Butyl acetate (AR grade)	Sigma
Chloroform	Fisons
Coomassie blue R250	Sigma
Diethanolamine	Sigma

Diethylaminoethyl (DEAE) - Sepharose CL6B	Pharmacia
4-N,N-dimethylaminoazobenzene-4'- isothiocyanate (DABITC)	Fluka
3,5-dinitrosalicylic acid	BDH
Dithiothreitol (DTT)	BRL
Ethanol	Fisons
Ethyl acetate	BDH
Diethyl-ether	Fisons
Ethidium bromide	Sigma
Ethylenediaminetetra-acetic acid disodium salt (EDTA) (AR Grade)	BDH
Glycerol	Fisons
Glycine	Sigma
Guanidine hydrochloride (sequencer grade)	Pierce
n-heptane	BDH
Hexane	BDH
Hydrochloric acid	Fisons
Iodoacetic acid	BDH
Magnesium acetate	Fisons
2-Mercaptoethanol	Sigma
Methanol	Fisons
Nitroblue tetrazolium	Sigma
Oligo-dT-cellulose	Sigma
Phenol	Fisons

Phenylisothiocyanate (PITC) (Sequencer grade)	Rathburn Chemicals Ltd.
Potassium chloride	Fisons
Potassium tartrate (AR grade)	BDH
Pyridine (AR grade)	BDH
Red Sepharose Cl-6B	Pharmacia
Sephadex G-50	Pharmacia
Sephadex G-75	Pharmacia
Sodium chloride	Fisons
Sodium citrate	Fisons
Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ )	BDH
Sodium dodecyl sulphate (SDS)	Sigma
di-Sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ )	BDH
Sodium hydroxide	Fisons
Sodium phosphate	Fisons
Starch	Sigma
Sucrose	Fisons
N,N,N',N'-Tetramethylene diamine (TEMED)	Sigma
Toluene (AR grade)	BDH
Trichloroacetic acid (TCA)	BDH
Trifluoroacetic acid (TFA) (Sequencer grade)	Rathburn Chemicals Ltd.
Triton X-100	BDH

Tris	Sigma
Tris-HCl	Sigma
Urea	BRL

### 2.3 Radiochemicals

Leucine [<sup>3</sup>H], (1mCi/ml), Amersham

[<sup>14</sup>C] Methylated proteins (0.833 µCi each proteins/ml in phosphate buffer, pH 7.2 containing 1% β-mercaptoethanol and 0.001 M EDTA), Amersham.

## METHODS

PURIFICATION, CHARACTERIZATION AND SEQUENCING OF A TRYPSIN INHIBITOR AND AN INSECT α-AMYLASE INHIBITOR /ENDOCHITINASE FROM SEEDS OF JOB'S TEARS (Coix lachryma-jobi)

### 2.4 Purification of a Trypsin Inhibitor

The purification of the inhibitor was carried out essentially as described by Ohtsubo et al. (1985).

Three hundred grams of Coix seeds were milled to a

fine powder. The defatted meal from Coix was homogenized with 1 liter of 50 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{K}_2\text{HPO}_4$  buffer, pH 7.5 containing 0.1 M NaCl (P-buffer) in a mixer for 10 min, and the precipitate was reextracted with the same solution. The combined extract was heated at 80°C for 10 min, then quickly cooled and centrifuged. Solid ammonium sulphate was slowly added to the supernatant to 40% saturation. After 2 h, the precipitate was removed by centrifugation, and solid ammonium sulphate was added to the supernatant to 95% saturation. After stirring for 2 h, the precipitate was collected by centrifugation and dissolved in 100 ml of distilled water and then dialyzed against distilled water at 4°C overnight.

After the precipitate was removed by centrifugation the dialyzate was applied to a column (2.6 x 31 cm) of DEAE-Sephrose CL6B equilibrated with P-buffer. The column was eluted with a linear gradient of 0.1-1.0 M NaCl (300 ml of each) in the same buffer. Fractions of 10 ml were collected at a flow rate of 27 ml/h. The elution profiles were followed by measurement of the absorbance at 280 nm of each fraction. The fractions containing trypsin inhibitory activity were pooled, desalted on distilled water and lyophilized.

The lyophilized crude protein was dissolved in 10 ml of P-buffer and applied to a gel filtration column (2.7 x 190 cm) of Sephadex G-75 equilibrated with the same buffer. Elution was with the same buffer. The elution profile was followed by measurement of the absorbance at 280 nm and by assaying for the

trypsin inhibitor activity of each fraction. The fractions containing trypsin inhibitory activity were pooled, desalted and lyophilized.

The partially purified trypsin inhibitor (35 mg) was dissolved in 6 M guanidinium chloride in 0.1% trifluoroacetic acid (1.5 ml) and injected onto a Vydac preparative C18 widepore reversed-phase column (25 cm x 22 mm, 218TP1022; Technicol, Stockport), in a Varian 5000 HPLC apparatus. The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (flow rate 10 ml/min).

## 2.5 Purification of an $\alpha$ -Amylase Inhibitor/Endochitinase

The 400 g of defatted seed powder was stirred for 5 h with 2 liters of 0.1 M HCl containing 0.15 M NaCl in a blender. The homogenate was centrifuged (10,000xg, 30 min, 4°C). The supernatant was adjusted to pH 7.0 by the dropwise addition of 1 M NaOH. The precipitated protein was removed by a further centrifugation (10,000xg, 10 min, 4°C). Ammonium sulphate was added to the supernatant to 60% saturation. The solution was left to stand overnight at 4°C and centrifuged (10,000xg, 30 min, 4°C). The precipitate was redissolved in 0.05 M Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl and dialyzed against the same buffer for 24 hs. In order to obtain a clear solution the insoluble protein was removed by centrifugation (10,000xg, 30 min, 4°C).

This clear solution was then applied to a column of

Red Sepharose CL-6B (8.5 x 3.0 cm) equilibrated with 0.05 M Tris-HCl (pH 7.0), containing 0.1 M NaCl as described by Kutty (1988). The column was washed with equilibration buffer until all unbound proteins was removed, and then eluted with the same buffer containing 3 M NaCl. Ten ml fractions were collected at a flow rate of 20 ml/h and analyzed for protein and  $\alpha$ -amylase inhibitory activity.

The active fractions were pooled, dialysed against distilled water and lyophilized. These were dissolved in 0.1 M trifluoroacetic acid containing 6 M guanidinium chloride and injected onto a Vydac preparative C18 wide-pore reversed phase column (25 cm x 22 mm, 218TP1D22; Technicol, Stockport), in a Varian 5000 HPCL apparatus. The column was eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 10 ml/min.

## 2.6 Assay of Trypsin Inhibitor Activity

Trypsin inhibitory activity was determined by measuring the inhibition of the hydrolysis of BAPNA by trypsin as proposed by Erlanger et al. (1961).

assay mixture - 0.5 ml trypsin (0.3 mg/ml in  
2.5 x 10<sup>-3</sup>M HCl)

0.5 ml inhibitor solution (in 0,05 M  
Tris-HCl pH 8.2)

2.0 ml BAPNA (1.25 x 10<sup>-3</sup> M in 0,05M  
\_\_\_\_\_ Tris-HCl pH 8.2) or 0.5ml of  
buffer alone (control)

Total volume: 3.0 ml

After incubating the enzyme with and without the inhibitor together for 5 min at 37°C, the substrate was added. After 10 min the reaction was stopped by the addition of 0.5 ml 30% acetic acid. The absorption of the solution was measured at 410 nm.

## 2.7 Assay of $\alpha$ -Amylase Inhibitor Activity

$\alpha$ -Amylase inhibitor activity was determined by the method of Bernfeld (1965).

assay mixture - 0.5 ml  $\alpha$ -amylase (1,25 g/ml in 0.05 M  
acetate buffer; pH 7.0  
0.5 ml starch solution (1% w/v )  
1.0 ml inhibitor solution (in 0.05 M  
acetate buffer, pH 7.0) or  
1.0ml of buffer alone

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Total volume      2.0 ml

After incubation of the enzyme and inhibitor together at 30°C for 25 min, the assay was started by the addition of the substrate. After 10 min the assay was terminated by the addition of 2.0 ml of dinitrosalicylate solution (prepared by dissolving 1.0 g of dinitrosalicylic acid in 20 ml 2 N NaOH and 50 ml H<sub>2</sub>O; 30 g of potassium tartrate (Rochelle salt) was added and the solution made up to 100 ml). The mixture was then

heated for 10 min in a boiling water bath and 10 ml of water were added to the solution; after cooling, the absorption at 530 nm was measured, and inhibition determined relative to the control.

## 2.8 Assay of Endochitinase Activity

[<sup>3</sup>H] colloidal chitin prepared as described by Leah et al. (1987) was incubated with the protein in 0.1 M citrate buffer, pH 5.0 for 15 mins at 37°C. The reaction (total volume 200 µl) was stopped by the addition of 300 µl of 10% (w/v) trichloroacetic and 100 µl of 1% (w/v) bovine serum albumin. The precipitated protein and undigested chitin were removed by centrifugation (10000xg for 5 min) and the released [<sup>3</sup>H] diacetyl chitobiose present in the supernatant determined by liquid scintillation counting. The enzyme activity was calculated from the specific activity of the [<sup>3</sup>H] chitin substrate.

## 2.9 SDS-Polyacrylamide Gel Electrophoresis

The SDS-polyacrylamide gel electrophoresis was done as described by Laemmli (1970), modified by the addition of 4 M urea in the stacking and separating gels (Bunce et al., 1985).

### Separating gel solution for 13% gels

40 ml 8 M urea/0.2 % SDS

18.8 ml 60% acrylamide/0.9% bisacrylamide

10 ml 3 M Tris-HCl pH 8.8  
3.4 ml distilled water  
6.8 ml 0.8% ammonium persulphate  
40  $\mu$ l TEMED

Stacking solution

8.0 ml 8 M urea/0.2% SDS  
4.0 ml 25% acrylamide/3.5% bisacrylamide  
4.0 ml 0.5 M Tris-HCl pH 6.8  
2.0 ml 0.8% ammonium persulphate  
20  $\mu$ l TEMED

Electrode buffer

56.8 g glycine (0.19 M)  
12.2 g Tris (0.0025 M)  
4.0 g SDS (0.1%)

To 4 l with distilled water, pH 8.8

The separating gel solution was poured to 1 cm below the comb and overlaid with water-saturated butan-1-ol. After polymerization the butan-1-ol was washed away and the top of the gel dried with Whatman n° 1 filter paper. The stacking gel was then poured and the comb inserted.

After loading the gel, it was run at 40 mA until the bromophenol blue had entered the separating gel and then at 60-65 mA until the bromophenol blue reached the bottom of the gel. The gel was stained overnight in 0.1% Coomassie Brilliant Blue

R250/10% TCA/40% methanol and destained in 10% (w/v) TCA.

### 2.10 Estimation of Molecular Mass by Gel Filtration

The molecular mass of the trypsin inhibitor was determined by gel filtration in Sephadex G-75, using P-buffer (see section 2.4).

The following proteins were used as markers for the determination of molecular mass: soybean Bowman-Birk inhibitor ( $M_r$  7 800), horse heart cytochrome c ( $M_r$  12 400), horse skeleton muscle myoglobin ( $M_r$  18 000) and beef pancreas chymotrypsinogen A ( $M_r$  25 700).

### 2.11 Estimation of Molecular Mass by High-Performance Gel Filtration

Molecular mass was estimated by high-performance gel filtration in an Ultropac TSK-G 2000 SW column (0.75 x 60 cm, LKB Produkter AB). The column was equilibrated and eluted with 6 M guanidinium chloride in 50 mM phosphate buffer, pH 6.5, containing 1 mM EDTA.

The column was calibrated using chymotrypsin ( $M_r$  25 300), trypsin ( $M_r$  24 500), soybean Kunitz inhibitor ( $M_r$  20 100), lysozyme ( $M_r$  14 000), wheat  $\alpha$ -amylase inhibitor ( $M_r$  13 400), soybean Bowman-Birk inhibitor ( $M_r$  7 800) and insulin ( $M_r$  5 800).

## 2.12 Isoelectric Focusing

Isoelectric focusing system used was carried out as described by Shewry et al. (1988). The 1% protein solution (w/v) was prepared in 10 mM glycine (pH adjusted to 8.0 with Tris), containing 6 M urea and 2% (v/v) 2-mercaptoethanol. The gel was prepared by dissolving 21.60 g of urea in 30 ml 10% acrylamide, 0.5% bisacrylamide, with 2% (w/v) ampholyte (pH range 3.5-10) and the final volume made up to 60 ml, to give a final urea concentration of 6M. Samples were separated using an LKB Multiphor System. Gels were washed with 10% (w/v) trichloroacetic acid to remove ampholyte and stained as described by Blakeslee and Borzi (1977).

In order to calculate the pH gradient across the gel, a strip containing separated protein was cut into 0.5 cm strips and placed in a test tube. 1.0 ml of distilled deionised water was added to each tube and the gel strips were macerated with a glass rod, left to stand for 1 h at 10°C and the pH measured.

## 2.13 Preparation of $\alpha$ -Amylase Activity from guts of Locusta migratoria migratorioides (African Migratory Locust)

Whole guts were dissected from mature larvae (nymphs) in distilled water at 4°C. Guts were then homogenised with a motorised teflon pestle. After centrifugation at 10000rpm for 30 min at 4°C, the supernatant was stored at -20°C. The extract from four guts was usually made up to a final volume of 1.0 ml.

## 2.14 Amino Acid Analysis

Duplicate samples of protein (100 µg) were hydrolysed under N<sub>2</sub> with 1.0 ml of constant boiling 5.9 M HCl for 21 h at 110°C. The hydrolysates were reduced to dryness and the amino acid composition determined using an LKB Alpha Plus amino acid analyser.

## 2.15 Sequence Determination

### 2.15.1 Reduction and S-Carboxymethylation

The inhibitors were reduced and S-carboxymethylated using a modification of the method of Crestfield et al. (1963) as described by Campos and Richardson (1983).

The protein (3-30 mg) was dissolved in 6 M guanidinium chloride buffered at pH 8.6 with 200 µl of 0.6 M Tris-HCl. 2-mercaptoethanol (30 µl) was added and the mixture left standing at room temperature under a constant stream of nitrogen, for 3 h. Then 100-300 µl of iodoacetic (0.268g/ml in 1.0 M NaOH) was added and the reaction kept in the dark for 30 mins. The reduced and s-carboxymethylated protein was recovered by desalting in the dark on a Bio-gel P2 column (1 x 20 cm) in distilled water. The eluted protein was lyophilized over solid NaOH.

### 2.15.2 Enzymatic Hydrolysis with Trypsin

Trypsin catalyses the hydrolysis of peptide bonds involving the carboxyl groups of lysine and arginine, except

where the following residue is proline. The trypsin used had been treated with TPCK to inhibit contaminating chymotrypsin-like activity (Inagami and Sturtvant, 1960).

The protein or peptide was dissolved in a minimal volume of 0.2 M N-ethylmorpholine buffer, pH 8.5 and trypsin (TPCK-treated) was added to give a 2% (w/w) enzyme/substrate ratio. After incubation for 2 h at 37°C, the reaction was stopped by lyophilisation.

#### 2.15.3 Enzymatic Hydrolysis with Chymotrypsin

Chymotrypsin cleaves peptides and proteins at the carboxyl side of tryptophan, phenylalanine, tyrosine, leucine and methionine, with occasional cleavage at other sites (e.g. histidine, asparagine). If the following residue is proline cleavage does not occur (Allen, 1981).

The condition of digestion were the same as those used for trypsin.

#### 2.15.4 Enzymatic Hydrolysis with Staphylococcus aureus (strain V-8) Protease

V-8 protease specifically cleaves peptides on the carboxyl side of glutamyl and aspartyl bonds if the digestion is performed in Na-phosphate buffer pH 7.8. The rate of digestion is low if the glutamic acid or aspartic acid is within three residues from either the N- or C- terminus of a peptide containing lysine or arginine and Glu-Pro and Asp-Pro peptide bonds are not cleaved (Austen and Smith, 1976; Croft,

1980). If the buffer is changed to either  $\text{NH}_4\text{HCO}_3$  (pH 7.8) or ammonium acetate (pH 4.0), cleavage only occurs at glutamyl residues.

The digestion was carried out with the protein or peptide being dissolved in a minimal volume of 0.1 M Na-phosphate buffer pH 7.8 and the enzyme was added to give a 2% (w/w) enzyme/substrate ratio. After incubation for 16 h at 37°C, the reaction was stopped by lyophilisation.

#### 2.15.5 Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

In order to purify the trypsin inhibitor or the  $\alpha$ -amylase inhibitor/endochitinase RP-HPLC was carried out using a Vydac preparative C18 wide-pore reversed-phase column (25cm x 22 mm, 218TP1022; Technicol, Stockport), in a Varian 5000 HPLC apparatus. Samples were dissolved in 6 M guanidinium chloride in 0.1% trifluoroacetic acid. The column was eluted with a stepped linear gradient of acetonitrile in 0.1% trifluoroacetic acid (flow rate 10 ml/min).

The mixtures of peptides produced by digestion with trypsin, chymotrypsin and S. aureus V-8 protease were fractionated and purified by RP-HPLC on a Vydac analytical reversed-phase column (25 cm x 4.6 mm, 218TP54, Technicol) in a Varian 5000 HPLC using variable gradients of 0-50% acetonitrile in 0.1% trifluoroacetic acid.

#### 2.15.6 DABITC/PITC Double Coupling Method

(Chang et al., 1978)

Samples of the peptide or protein were placed in a glass tube (0.5 x 3.0 cm) fitted with a ground glass stopper and dissolved in 80  $\mu$ l of 50% (v/v) aqueous pyridine. 40  $\mu$ l of the DABITC solution (2.82 mg/ml in pyridine) were added, the tube flushed with nitrogen, the contents mixed on a whirlmixer and incubated at 52°C for 50 min. PITC (10  $\mu$ l) was then added, the mixture flushed again with nitrogen and incubated at 52°C for 30 mins. The excess reagents were removed by extracting the mixture three times with a 2:1 (v/v) solution of heptane/ethyl acetate. The organic phase was separated in each case by centrifugation, removed by pipette and discarded. The aqueous phase was dried in vacuo over solid NaOH. The extraction of the DABTZ-amino acid was done by adding to the residue 50  $\mu$ l of water and 200  $\mu$ l of butyl acetate. After mixing on a whirlmixer for three seconds, the contents were centrifuged for 30 seconds at 1000 g. The upper butyl acetate layer was removed with a pipette and the peptide or protein in the aqueous phase was dried down in vacuo over solid NaOH and subjected to the next degradation cycle.

The butyl acetate extract was dried in vacuo over solid NaOH and the residue was dissolved in 50  $\mu$ l of 50% aqueous TFA. Conversion of the thiazolinones of the amino acids into the thiohydantoin was carried out by heating at 80°C for 10 min. The sample was dried in vacuo over solid NaOH and redissolved in a suitable volume (5-30  $\mu$ l) of 95% (v/v)

ethanol.

The identification of the DABTH-aminoacid was done by thin layer chromatography (TLC) on small (3x3cm) polyamide sheets. The synthetic marker 4-N,N- dimethylaminoazobenzene-4'-thiocarbamyl (DABTC)-diethylamine was used as a marker and spotted in the same position as the ethanol extract. The first dimension separation was done using an acetic acid/water (1:2, v/v) solution and the second dimension separation with a toluene/n-hexane/acetic acid solution (2:1:1, v/v/v). After TLC the dried sheets were exposed to HCl vapour leading to the temporary formation of the characteristic colours of the DABTH-amino acids. The DABTH- amino acids were identified by comparing the positions of the coloured spots with the blue marker.

#### **2.16 Comparison of Amino Acid Sequences**

The amino acid sequence of the Bowman-Birk trypsin inhibitor from seeds of Coix was compared with those of other proteins stored in a database (US National Biomedical Research Foundation, 1986) and the sequences of other inhibitors more recently obtained, by computer analysis using the FASTP and RDF programmes as described in Lipman and Pearson (1985).

#### **2.17 Analysis of Protein Secondary Structure and Hydropathy by Predictive Methods**

The profile of hydropathy of the Bowman-Birk trypsin

inhibitor from seeds of Coix was predicted from the amino acid sequence by the method of Kyte and Doolittle (1982) using the computer programme of Krystek et al. (1985).

The secondary structure of the protein above was predicted by the computer method of Garnier et al. (1978).

## MOLECULAR METHODS-ISOLATION AND CHARACTERIZATION OF mRNA FRACTIONS

### 2.18 Preparation of Antisera

Samples of the purified trypsin and  $\alpha$ -amylase inhibitors from Coix were suspended in PBS (20 mM  $N_2HPO_4$ , 0.85 M NaCl, HCl to pH 7.4) to give a concentration of 1 mg/ml. The protein solution was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected into the thigh muscle of a rabbit (1 ml/leg). The rabbits were New Zealand White, between 6 months and 1 year in age. Subsequent booster injections were given after 4 weeks using Freund's incomplete adjuvant.

The rabbits were bled every 2 weeks. The blood was allowed to clot at 37°C for 1 h in 30 ml centrifuge tubes, the clot removed from the tube and the remaining suspension left at 40°C for 5-16 h. The suspension was centrifuged at 5000 rpm for 10 min and the serum removed and frozen at -20°C.

## 2.19 Immuno-Assay

This is a method for detecting proteins following immuno-blotting. Phosphatase-conjugated antibodies are used to identify immobilized antigen-antibody complexes. This protocol was modified from the method of Avrameas and Guilbert (1971).

The proteins dissolved at 0.4 mg/20  $\mu$ l in PBS (see section 2.18) was spotted on a nitrocellulose filter (Hybond-C extra, from Amersham) and allowed to dry. The filters were then rinsed in assay buffer (0.9% NaCl, 10 mM Tris pH 7.4, 0.05% Triton X-100) plus 5% of dried skimmed milk powder (from Waitrose) on a shaker for 2 h. The filters were then placed in plastic bags containing either the Coix  $\alpha$ -amylase inhibitor antiserum or the sugar beet chitinase antiserum or the wheat germ chitinase antiserum. After shaking the plastic bags overnight at 4°C the filters were rinsed briefly in assay buffer plus dried skimmed milk and left for 1 h in this same solution. The filters were removed from the bags and placed on plates. Protein-A-Alkaline Phosphatase (10  $\mu$ l/10 ml assay buffer plus milk) was added to the plates and shaken for 2 h. The filters were then rinsed again in buffer plus milk for 3 x 10 min, in diethanolamine buffer (100 mM diethanolamine, pH 9,8) for 1 x 10 min and incubated with the "assay mix" (10 mg nitroblue tetrazolium in diethanolamine buffer; 5 mg 5-bromo-4-chloro-3-indolyl phosphate to 100 ml with diethanolamine buffer more 400  $\mu$ l of 1 M MgCl<sub>2</sub>) until the spots became blue (between 5 and 60 mins). Finally the filters were washed with distilled water and dried in air.

## 2.20 Isolation of Polysomes and RNA

Whole seeds of field grown Job's Tears (Coix lachryma jobi) were harvested according the following criteria: presence of pollen, seeds still soft and green but starting to show the brown colour that characterizes the mature seed, and between 20 and 30 days after anthesis. The seeds were collected, washed quickly with SDW, frozen in liquid nitrogen and stored in 50 g batches at  $-80^{\circ}\text{C}$ .

Sterile glassware and solutions were used in all procedures for the isolation of RNA. Unless otherwise stated all procedures were carried out at  $4^{\circ}\text{C}$ .

### 2.20.1 Polysome isolation

Polysomes were isolated according to the method of Fox et al., (1977). 50 g of frozen seeds were ground to a fine powder in a coffee mill. The powder was added to 140 ml of grinding buffer B and Triton X-100 (2.1 g/70 ml grinding buffer B) in a blender, homogenized for one minute and centrifuged in 100 ml glass centrifuge tubes at 1000rpm for 5 min at  $2^{\circ}\text{C}$  to pellet unbroken cells and cell debris. The supernatant was transferred to 50 ml centrifuge tubes and spun for 25 min at 17000rpm at  $2^{\circ}\text{C}$ . The supernatant was layered over a 54% (w/v) sucrose cushion (4.0 ml) in polycarbonate tubes and spun at 50000rpm for 4 h at  $2^{\circ}\text{C}$ . After centrifugation the supernatant was removed with a water-vacuum pump. The polysomes were resuspended in 10 ml of phenol extraction buffer and stored overnight at  $-20^{\circ}\text{C}$ .

### 2.20.2 Isolation of Poly (A<sup>+</sup>)-rich mRNA

The polysomes resuspended in phenol extraction buffer were extracted with phenol/chloroform (Wienard and Feix, 1978) followed by centrifugation at 1000rpm for 5 min at 18°C. The aqueous layer was collected and the organic layer re-extracted with phenol extraction buffer and centrifuged for 5 min at 1000rpm at 18°C. After centrifugation the two aqueous layers were pooled and extracted with phenol/chloroform. The aqueous layer was removed after centrifugation for 5 min at 1000rpm at 18°C and the bottom organic phase was re-extracted with phenol extraction buffer. Finally, the pooled aqueous layers were extracted with an equal volume of chloroform.

The RNA present in the aqueous layer was precipitated with 4 M ammonium acetate and ethanol overnight at -20°C. The supernatant was poured off and the pellet covered with 75% ethanol. After centrifugation for 10 min at 10000rpm and 2°C the 75% ethanol was poured off and the pellet dried under vacuum. The pellet was dissolved in 1.0 ml of SDW and stored at -80°C.

Poly (A<sup>+</sup>)-rich mRNA was prepared by oligo-dT chromatography (carried out at RT) using the method of Bantle *et al.* (1976). Oligo-dT cellulose was prepared by washing with 0.1 M NaOH and then with binding buffer. The RNA was heated to 65°C for 5 mins, cooled rapidly on ice, added to the oligo-dT-cellulose and stirred gently for 45 mins. The column was poured and washed with binding buffer until the optical density at 260 nm was zero. The column was then washed with washing buffer. To

remove the poly (A<sup>+</sup>)-rich mRNA the column was washed with elution buffer. This buffer does not contain NaCl which stabilises the binding of the poly (A<sup>+</sup>)-rich mRNA to the oligo-dT. The polyadenylated RNA rich fractions were precipitated with 4 M ammonium acetate and ethanol overnight at -20°C. After centrifugation for 25 min at 10000rpm and 2°C the supernatant was poured off and the pellet dried under vacuum. The pellet was then washed with 75% ethanol, spun for 10 min at 10000rpm at 2°C, the ethanol poured off, the pellet dried under vacuum and dissolved in 100 µl of SDW and stored at -80°C.

Solutions:

<u>Grinding buffer A</u> (stored at 4°C)	<u>500 ml</u>
0.2 M Tris-HCl	12.11 g
0.12 M KCl	4.48 g
0.05 M Mg acetate	5.37 g
Autoclaved	pH 9.0
<u>Grinding buffer B</u> (made up fresh)	<u>500 ml</u>
Grinding buffer A	500 ml
Sucrose	43.0 g
DTT	0.40 g
2'.3'. AMP	0.40 g
<u>54% sucrose solution</u> (made up fresh)	<u>50 ml</u>
Sucrose	27.00 g
DTT	0.04 g
2',3',AMP	0.04 g
Grinding buffer A	23 ml

<u>Phenol extraction buffer</u> (stored at RT)	<u>200 ml</u>
0.2 M Tris-HCl	4.84 g
0.1 M NaCl	1.17 g
0.01 M EDTA	0.75 g
Autoclaved	pH 8.8

Buffers for Oligo-dT-cellulose column(stored at 4°C):

<u>Binding buffer</u>	<u>400 ml</u>
10.0 mM Tris-HCl	0.48 g
0.1 M NaCl	9.35 g
1.0 mM EDTA	0.15 g
0.1 % (w/v) SDS	0.40 g
Autoclaved	pH 8.5

<u>Elution buffer</u>	<u>200 ml</u>
10.0 mM Tris-HCl	0.24 g
1.0 mM EDTA	0.075g
0.1 % (w/v) SDS	0.20 g
Autoclaved	pH 8.5

2.21 In vitro Translation of Poly (A<sup>+</sup>)-rich mRNA Using the  
Wheat Germ System

Poly (A<sup>+</sup>)-rich mRNA was translated using wheat germ cell-free extracts supplemented with [<sup>3</sup>H] leucine as described by Kreis et al., (1983).

Basic Mixture:

5  $\mu$ l ATP/CP (40 mM ATP neutralised in Tris pH  
7.6)

5  $\mu$ l 12 mM spermidine

2  $\mu$ l 5 mM GTP

2  $\mu$ l 200 mM DTT

2  $\mu$ l CPK (120 U/mg, 5mg/ml in 50% glycerol)

2  $\mu$ l amino acids (- leucine), 5 mM each in 5 mM  
DTT

25  $\mu$ l salts n° 6 ( $Mg^2/K$ )

50  $\mu$ l [ $^3H$ ] leucine (1 mCi/ml)

32  $\mu$ l SDW

Total volume 125  $\mu$ l

Reaction Mixture:

12.5  $\mu$ l basic mixture

1.4  $\mu$ l poly ( $A^+$ )-rich mRNA

2.5  $\mu$ l wheat germ

3.6  $\mu$ l SDW

Total Volume 20  $\mu$ l

The reaction mixture was vortexed gently and centrifuged for 2 seconds to ensure the reactants were mixed and then incubated at 28°C for 40 mins. 2  $\mu$ l of a solution of unlabelled 1% leucine was added and 2  $\mu$ l aliquots of the mixture were spotted onto 2 cm discs of Whatman No. 1 filter paper and allowed to dry. The filters were placed in 100 ml of

10% TCA (w/v) and boiled for 20 min. The filters were left to stand for 20 min and then rinsed with 100 ml cold 10% (w/v) TCA followed by 100 ml 95% ethanol and 100 ml diethyl ether and finally allowed to dry. The filters were placed in vials with 3 ml non aqueous scintillant and counted by liquid scintillation. To the remaining reaction mixture was added 20  $\mu$ l of gel loading buffer (4 M urea/2% (w/v) SDS /2% (v/v) 2-mercaptoethanol/0.125 M Tris-HCl pH 6.8, 0.002% bromophenol blue in 25% (w/v) glycerol). The mixture was heated to 65 C for 20 mins, loaded on a 13% urea/SDS polyacrylamide gel (see Section 2.9 including 0.03% and 0.056% of DTT in the separating and stacking gels respectively) and after running the gel was subjected to autoradiography

## 2.22 Immunoprecipitation of in vitro Translation Products

Immunoprecipitation of in vitro translation products using antibodies raised against the  $\alpha$ -amylase inhibitor/endochitinase, the sugar beet endochitinase and the wheat germ endochitinase were performed using the method described by Jonassen et al., (1981).

### Buffer A (Immunoprecipitation buffer):

50 mM Tris-HCl

0.15 M NaCl

2% Triton X-100

10.0 mM Leucine                      pH 7.7

Buffer B:

10.0 mM Tris pH 7.5

Four in vitro translation reaction mixtures were diluted to volumes of 600  $\mu$ l with buffer A in 1.5 ml eppendorf tubes and shaken gently for 30 min on a multimix at RT. The tubes were then centrifuged at 12000rpm for 15 min to remove insoluble material. To each supernatant was added 40  $\mu$ l of the antiserum to the sugar beet or the wheat germ endochitinase or 100  $\mu$ l of the non-immune serum or the antiserum to the Coix - amylase inhibitor/endochitinase. The tubes were shaken for 2 h at RT, after which 100  $\mu$ l of Protein A Sepharose (43 mg/ml buffer A with 0.5% BSA) was added and the tubes were shaken again for 2 h at RT. The tubes were then spun at 15000rpm in a microfuge for 15 min and the pellet washed x5 with buffer A and x3 with buffer B. Each pellet was resuspended in 20  $\mu$ l loading buffer (containing 8M urea, 4% (v/v)SDS, 4% 2-mercaptoethanol in Tris-HCl, pH 6.8 and 10ul of a solution of 0.004% bromophenol blue in 50% glycerol). The mixture was then heated to 65°C for 20 min, spun in a microfuge for 5 min and loaded onto a 13% SDS polyacrylamide gel (see section 2.9 including 0.03% and 0.56% of DTT in the separating and stacking gels respectively).

## 2.23 Cleavage of DNA with Restriction Endonucleases

Plasmid DNA was digested with 1-5 units of

restriction enzyme per  $\mu\text{g}$  DNA. The reaction volume was generally 10  $\mu\text{l}$  but varied with the quantity and concentration of DNA to be digested. Reaction buffers and temperatures were as specified by the manufacturer.

#### 2.24 Agarose Gel Electrophoresis of DNA (Maniatis et al. 1982)

This method was used with minigels in order to characterize DNA. The concentration of agarose in the gel depends on the type of agarose used and the size of fragments to be separated. For most purposes a 1% gel agarose was used. The agarose was melted in 1x TEA buffer in a microwave oven and the gel cast in the tray of a Biorad mini-gel apparatus using 30 ml gel volume.

##### 20x TEA buffer:

Tris	96.8 g
Glacial acetic acid	22.8 ml
EDTA	3.72 g
pH 8.0	1L

After setting, the tray was placed in the apparatus and covered with 1x TEA buffer. A fifth volume of 5x gel loading buffer (25% (v/v) glycerol, 25%(v/v) saturated solution of bromophenol blue in 1x TEA buffer) was added to the DNA solution before loading. The voltage and time of electrophoresis was varied but the current was not allowed to

exceed 30 mA to prevent overheating.

To stain the DNA the gel was transferred to a solution of 1 µg/ml of ethidium bromide. After 100 min the mini-gel was rinsed then destained for the same time in distilled water. The gel was viewed using a long wavelength (300-360 nm) UV transilluminator and photographed using a Polaroid Land Camera with a red filter.

CHAPTER 3

RESULTS AND DISCUSSION

PURIFICATION, CHARACTERIZATION AND SEQUENCING OF A  
TRYPSIN INHIBITOR FROM SEEDS OF JOB'S TEARS (Coix lachryma-  
jobi)

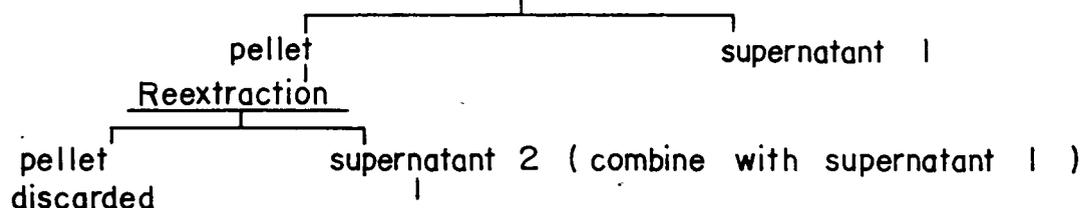
3.1 Purification and Characterization

The trypsin inhibitor was purified using a number of steps (from Extraction until Gel Filtration chromatography on Sephadex G-75) from the purification method of Ohtsubo et al. (1985), followed by preparative reversed-phase high performance liquid chromatography (RP-HPLC). The flow chart in Fig. 1 shows the procedures carried out during the purification of the trypsin inhibitor.

The elution profile of the DEAE-Sepharose CL6B column (Fig. 2) shows that the inhibitor peak appeared at a NaCl concentration below 0.5 M, as reported by Ohtsubo et al. (1985). The elution profile of the gel filtration chromatography yielded a major peak of trypsin inhibitory activity together with a number of other minor protein peaks (Fig. 3). The final purification step, preparative RP-HPLC yielded two peaks of trypsin inhibitory activity, one major (TI1) and one minor (TI2), together with a number of other protein peaks which were devoid of inhibitor activity (Fig. 4). The protein in the major peak (TI1) was eluted at an acetonitrile concentration of 24 % which is very similar to the value reported for the single-headed ( 7kDa) wheat germ trypsin

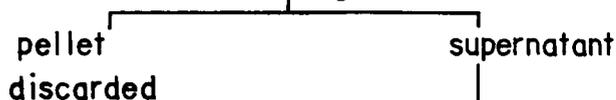
## Extraction

300 g Coix flour +  
1 l P- buffer (see section 2.4)  
1 h at RT  
centrifuge



## Heat Treatment

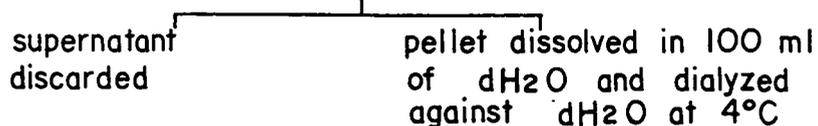
10 min at 80°C  
cooled in ice-bath  
centrifuge



Ammonium Sulphate 0-40%  
centrifuge



Ammonium Sulphate 40-95%  
centrifuge



Ion Exchange Chromatography in DEAE-Sepharose CL6B (Fig. 2)

Gel Filtration Chromatography on Sephadex G-75 (Fig. 3)

Reversed-Phase High Performance Liquid Chromatography (Fig. 4)

Figure 1. A flow chart showing the purification of a trypsin inhibitor from Coix seeds.

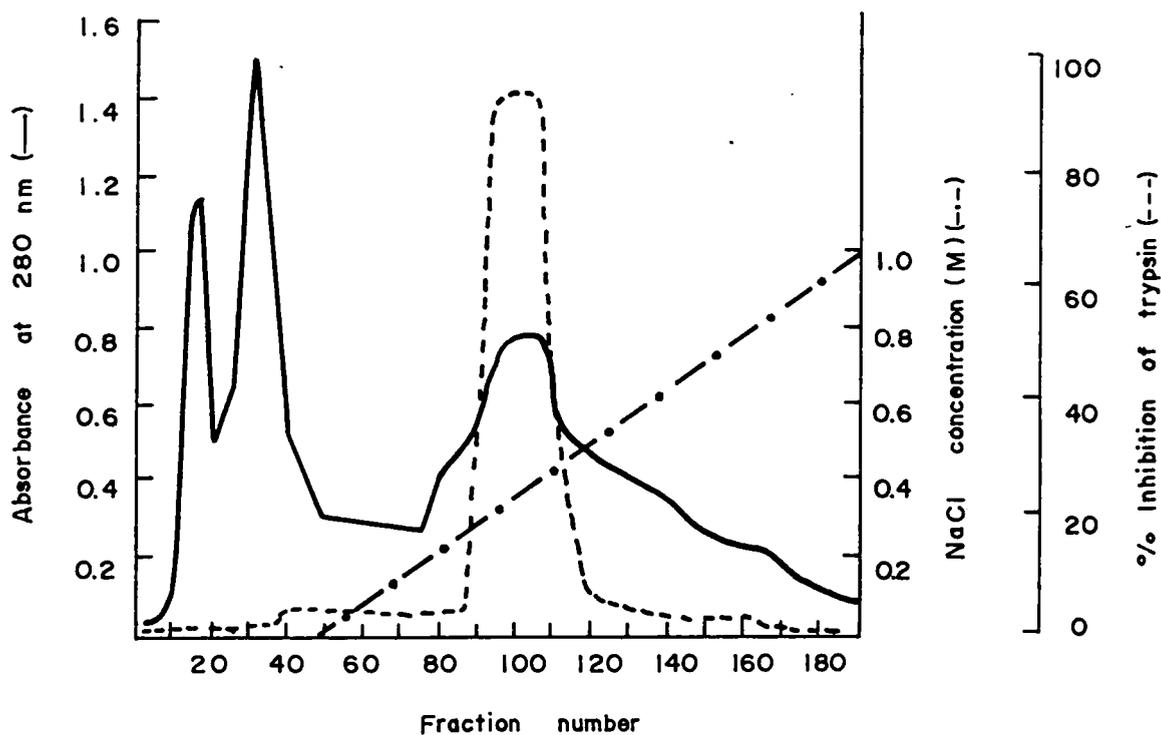


Figure 2. Ion-exchange chromatography of the Coix trypsin inhibitor (40-90% ammonium sulphate fraction) on DEAE-Sepharose CL6B (2.6 x 31 cm) in P-buffer (see section 2.4), eluted with a linear gradient of 0.1-1.0M NaCl.

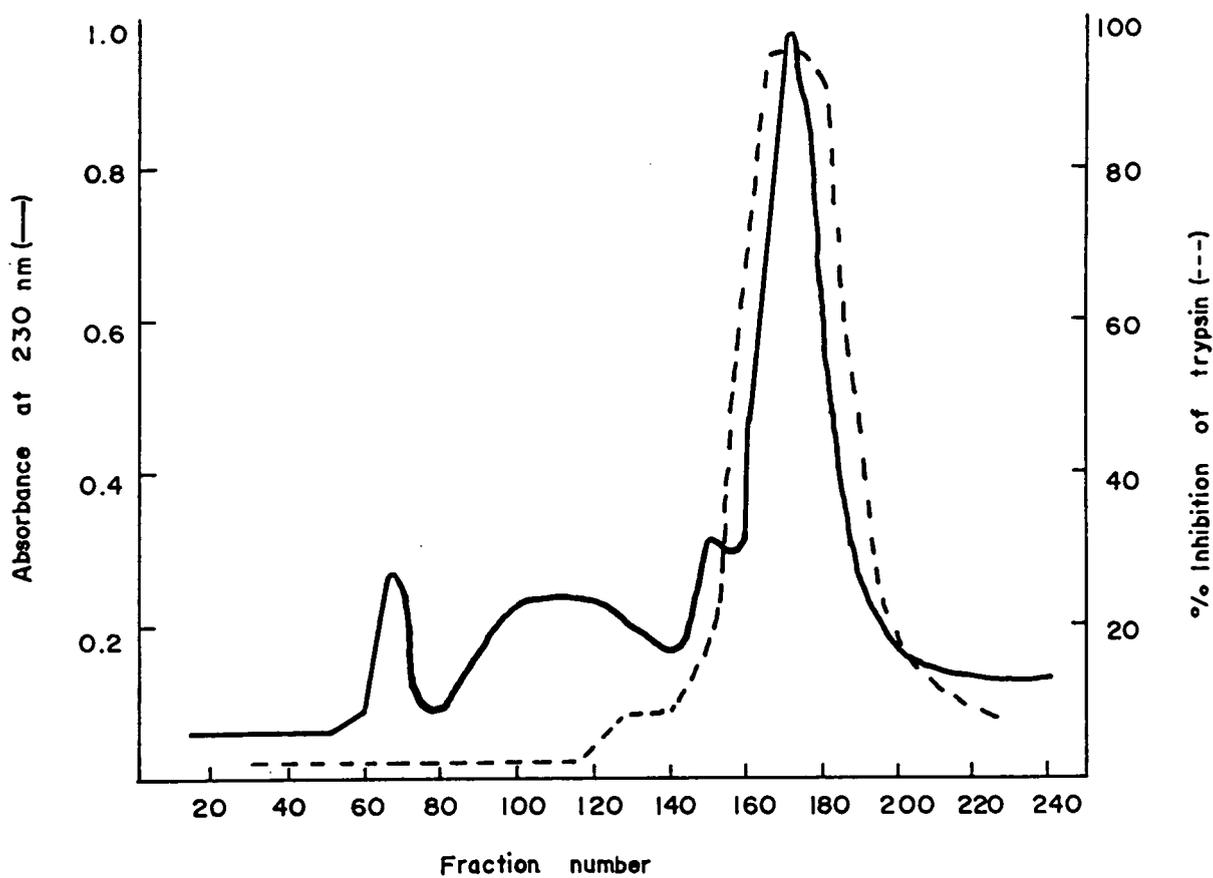


Figure 3. Gel filtration chromatography of the Coix trypsin inhibitor after DEAE-Sepharose CL6B on Sephadex G-75 (2.6 x 190 cm) in P- buffer (see section 2.4).

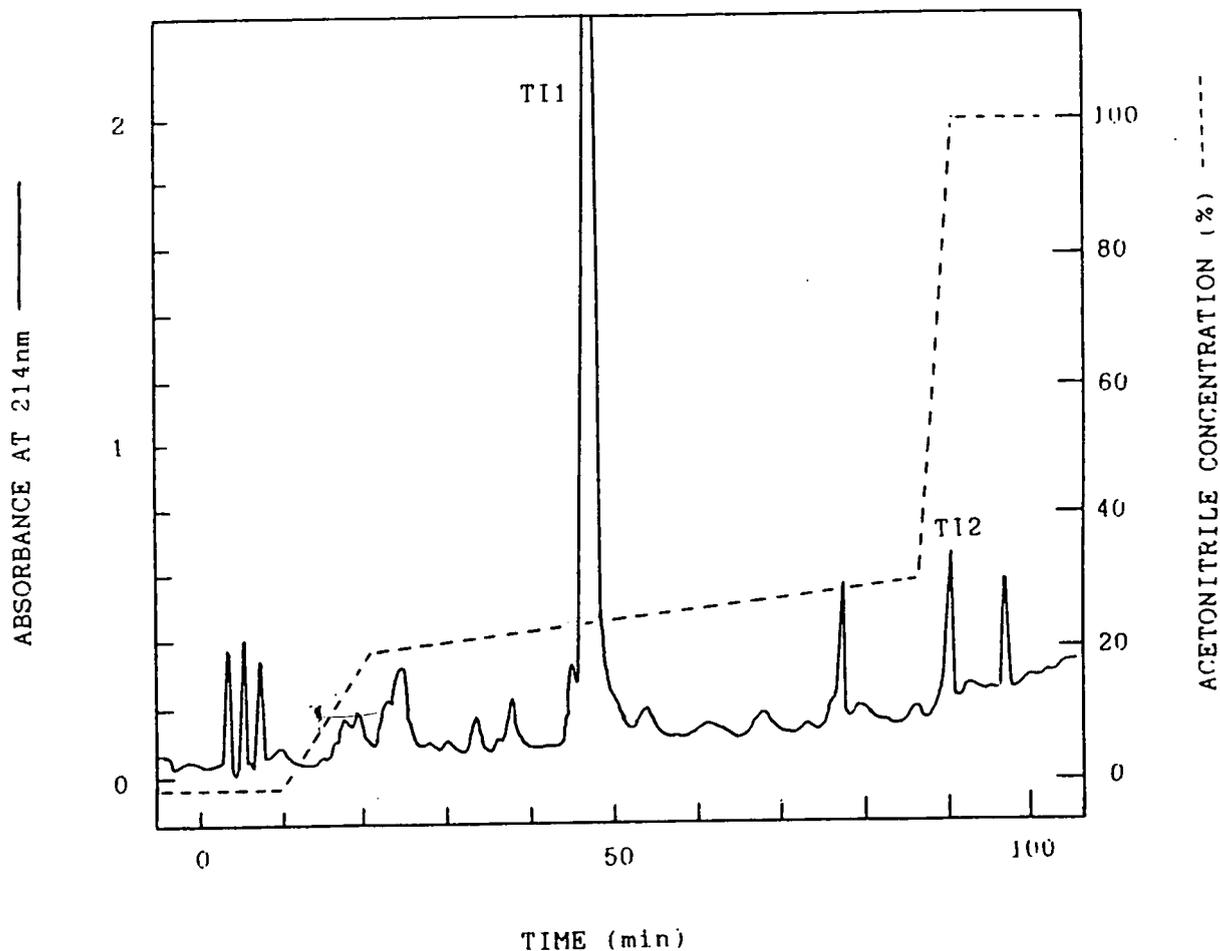


Figure 4. Reversed-phase chromatography of the Coix trypsin inhibitor ( after Gel Filtration on Sephadex G-75 ) on a preparative C18 column (25 cm x 22 mm), eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 10ml/min

inhibitor isolated by Odani et al. (1986). The major peak (TI1) was used for all subsequent analyses.

### 3.1.1 Determination of Molecular Mass of Inhibitor TI1

During gel filtration on Sephadex G-75 the elution volume of inhibitor TI1 was very similar to that observed for the 7.8 kDa soybean Bowman-Birk proteinase inhibitor (Fig. 5). When subjected to high-performance gel filtration on a TSK-G 2000SW column in 6M guanidine HCl the purified protein TI1 was shown to be homogeneous with a molecular mass of 7kDa. However, pretreatment with either dithiothreitol or 2-mercaptoethanol led to the appearance of a form of 14.5 kDa and also larger presumably aggregated forms. The inhibitor also behaved anomalously on SDS-PAGE giving a single narrow band of apparent molecular mass 11 kDa in the absence of reducing agent, and broader more diffuse bands (17-20 kDa) after treatment with 2-mercaptoethanol. The higher values of molecular mass observed after treatment with reducing agents probably resulted from re-oxidation to form inter-chain disulphide bonds, and confirm the observations of Ohtsubo et al. (1985) that the Coix inhibitor had a similar mobility to cytochrome c (12.5 kDa) on SDS-PAGE, but eluted after this protein during gel filtration on Sephadex G-100.

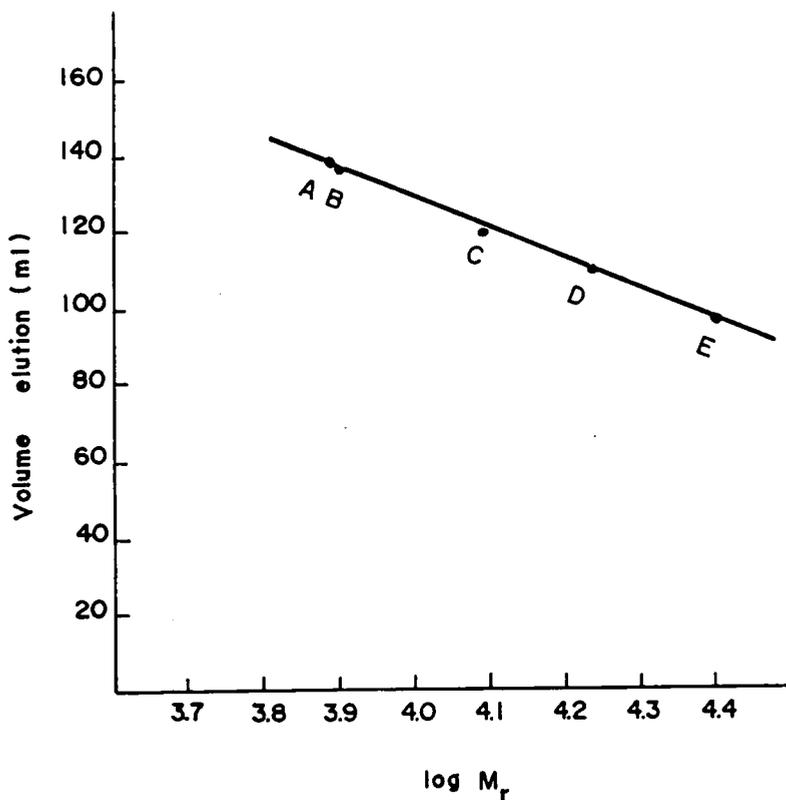


Figure 5. Molecular mass estimation of Coix trypsin inhibitor. Results of the gel filtration chromatography on Sephadex G-75 (142 x 1.5 cm) in P-buffer (see section 2.4). A, Coix trypsin inhibitor; B, soybean Bowman-Birk inhibitor ( $M_r$  7 800); C, cytochrome c ( $M_r$  12 400); D, myoglobin ( $M_r$  18 000); E, chymotrypsinogen ( $M_r$  25 700).

### 3.2 Determination of the Complete Amino Acid Sequence

#### 3.2.1 Enzymatic Digestion with Trypsin

2.5 mg of the reduced and carboxymethylated trypsin inhibitor were digested with trypsin, under the conditions described in section 2.15.2. The peptides obtained were purified on a Vydac analytical reversed-phase column. The sequences of the peptides obtained, the acetonitrile concentrations at which they were eluted and their positions in the sequence are given in Table III.

#### 3.2.2 Enzymatic Digestion with Chymotrypsin

2.5 mg of the reduced and carboxymethylated trypsin inhibitor were digested with chymotrypsin under the conditions described in section 2.15.3. The peptides obtained were purified on a Vydac analytical reversed-phase column. The sequences of the peptides obtained, the acetonitrile concentrations at which they were eluted and their position in the sequence are given in Table IV.

#### 3.2.3 Enzymatic Digestion with S. aureus Protease (strain V8)

2.5 mg of the reduced and carboxymethylated trypsin inhibitor were digested with S.aureus V8 protease, under the conditions described in section 2.15.4. The peptides obtained

**TABLE III - Peptides obtained by cleavage of the Coix trypsin inhibitor with trypsin**

Peptide nº and Position in Sequence	Acetonitrile Concentration (%)	Sequence
T1 (5-17)	22	Arg-Pro-Trp-Glu-Cys-Cys-Asp- -Ile-Ala-Met-Cys-Thr-Arg-
T2 (18-24)	17	Ser-Ile-Pro-Pro-Ile-Cys-Arg-
T3 (25-31)	10	Cys-Val-Asp-Lys-Val-Asp-Arg-
T4 (32-46)	11	Cys-Ser-Asp-Ala-Cys-Lys-Asp- -Cys-Glu-Glu-Asp-Asn-Arg-
T5a (47-64)	23	His-Val-Cys-Phe-Asp-Thr-Tyr- -Ile-Gly-Asp-Pro-Gly-Pro-Thr- -Cys-His-Asp-Asp-
T5b (47-53)	19	His-Val-Cys-Phe-Asp-Thr-Tyr-
T5c (54-64)	16	Ile-Gly-Asp-Pro-Gly-Pro-Thr- -Cys-His-Asp-Asp-

**TABLE IV - Peptides obtained by cleavage of the Coix trypsin inhibitor with chymotrypsin**

Peptide nº and Position in Sequence	Acetonitrile Concentration (%)	Sequence
C1a (1-7)	15	Gly-Asp-Glu-Lys-Arg-Pro-Trp-
C1b (2-7)	14	Asp-Glu-Lys-Arg-Pro-Trp-
C2 (8-14)	16	Glu-Cys-Cys-Asp-Ile-Ala-Met-
C3a (17-39)	20	Arg-Ser-Ile-Pro-Pro-Ile-Cys- -Arg-Cys-Val-Asp-Lys-Val-Asp- -Arg-Cys-Ser-Asp-Ala-Cys-Lys- -Asp-
C3b (18-31)	25	Ser-Ile-Pro-Pro-Ile-Cys-Arg- -Cys-Val-Asp-Lys-Val-Asp-Arg-
C3c (32-48)	19	Cys-Ser-Asp-Ala-Cys-Lys-Asp- -Cys-Glu-Glu-Thr-Glu-Asp-Asn- -Arg-His-Val-
C4a (53-64)	9	Tyr-Ile-Gly-Asp-Pro-Gly-Pro- -Thr-Cys-His-Asp-Asp-
C4b (60-64)	3	Cys-His-Asp-Asp-

were purified on a Vydac analytical reversed-phase column. The sequences of the peptides obtained, the acetonitrile concentrations at which they were eluted and their position in the sequence are given in Table V.

#### 3.2.4 Sequence Analysis of the Coix Trypsin Inhibitor

The complete amino acid sequence of the Coix trypsin inhibitor TI1 is shown in Fig. 6 together with details of the overlapping peptides from which it was deduced.

The protein contains 64 amino acid residues, corresponding to a  $M_r$  of 7 262 which is in good agreement with the molecular mass determined by high-performance gel filtration (7kDa).

It should be noted that the N-terminal glycine residue was missing in about 30% of the chains of the intact polypeptide, but no other microheterogeneity was detected.

#### 3.2.5 Sequence Comparison

Alignment of the complete amino acid sequence of the Coix trypsin inhibitor (Fig. 7) shows clear sequence homologies with the rice bran inhibitor (Tashiro et al., 1987), wheat germ inhibitors (Odani et al., 1986), foxtail millet inhibitor (Tashiro et al., 1990) and Bowman-Birk proteinase inhibitors from a number of legumes (Odani and Ikenaka, 1977; Norioka and

**TABLE V - Peptides obtained by cleavage of the Coix trypsin inhibitor with S.aureus (Strain V-8) protease**

Peptide nº and Position in Sequence	Acetonitrile Concentration (%)	Sequence
V1a (1-8)	15	Gly-Asp-Glu-Lys-Arg-Pro-Trp- -Glu-
V1b (2-8)	16	Asp-Glu-Lys-Arg-Pro-Trp-Glu-
V1c (4-16)	26	Lys-Arg-Pro-Trp-Glu-Cys-Cys- -Asp-Ile-Ala-Met-Cys-Thr-
V2a (9-26)	25	Cys-Cys-Asp-Ile-Ala-Met-Cys- -Thr-Arg-Ser-Ile-Pro-Pro-Ile- -Cys-Arg-Cys-Val-
V2b (12-32)	24	Ile-Ala-Met-Cys-Thr-Arg-Ser- -Ile-Pro-Pro-Ile-Cys-Arg-Cys- -Val-Asp-Lys-Val-Asp-Arg-Cys-
V3a (42-63)	17	Thr-Glu-Asp-Asn-Arg-His-Val- -Cys-Phe-Asp-Thr-Tyr-Ile-Gly- -Asp-Pro-Gly-Pro-Thr-Cys-His- -Asp-
V3b (44-51)	23	Asp-Asn-Arg-His-Val-Cys-Phe- -Asp-
V3c (52-64)	18	Thr-Tyr-Ile-Gly-Asp-Pro-Gly- -Pro-Thr-Cys-His-Asp-Asp-



Figure 6. Amino acid sequence of the major trypsin inhibitor TII from seeds of Coix. T, tryptic peptides; C, chymotryptic peptides; V, peptides from digestion with S. aureus V8. Regions of peptides sequenced by the DABITC method ( - - - ); blank spaces, residues were not sequenced or yielded unsatisfactory results; →→, residues determined by direct DABITC sequencing of the intact reduced and S-carboxymethylated protein.

Figure 7. Homology of the amino acid sequences of the Bowman-Birk family of proteinase inhibitors from legumes and cereals: 1) inhibitor CII from soybean (Odani and Ikenaka, 1977); 2) inhibitor AII from peanut (Norioka and Ikenaka, 1983); 3) adzuki bean (Yoshikawa et al., 1979); 4) Macrotyloma axillare (Joubert et al., 1979); 5) Vicia angustifolia (Shimokawa et al., 1984); 6) mung bean (Wilson and Chen, 1983); 7) faba beans (Asao et al., 1991); 8) alfafa leaf (Brow et al., 1985); 9) rice bran residues 1-69 (Tashiro et al., 1987), residues 70-133 are an internal duplication homologous with residues 3-69, and are shown as 9\*; 10) wheat germ II-4 incomplete sequence (Odani et al., 1986); 11) wheat I-2b incomplete sequence (Odani et al., 1986); 12) foxtail millet (Tashiro et al., 1990); 13) Coix trypsin inhibitor. Only the numbering of the soybean (top line) and the Coix (bottom) sequences are shown. The sequences are aligned for maximum homology, resulting in some gaps ( - ) which may represent insertions/deletions. Arrows indicate the position of the reactive sites determined for the legume inhibitors. The cysteine residues are boxed to facilitate comparisons.

- |                       |                           |
|-----------------------|---------------------------|
| 1. SOYBEAN CII        | S D H S S S D D E         |
| 2. PEANUT AII         | E A S S S S               |
| 3. ADZUKI BEAN        | S G H H E D T T D E P S E |
| 4. MACROTYLOMA DE3    | D H H H S T D E P S E     |
| 5. VICIA ANGUSTIFOLIA | G D D                     |
| 6. MUNG BEAN          | S S H H H D S S D E P S E |
| 7. FABA BEAN          | G D D                     |
| 8. ALFAFA LEAF        |                           |
| 9. RICE BRAN          | M E                       |
| 10. WHEAT GERM II-4   | A T                       |
| 11. WHEAT GERM I-2b   | A A K K                   |
| 12. FOXTAIL MILLET    | S G E                     |
| 13. COIX              | G D E K                   |

	10	20	↓	30
1	- S S K P	C C D L	C M C T A S M	- - P P - - Q C H C A D I R L N
2	- D D N V	C C N G	C L C D R R A	- - P P Y F E C V C Y D T F D H
3	- S S K P	C C D Q C	- C T K S M	- - P P - - K C R C S D I R L D
4	- S S K P	C C D E C	A C T K S I	- - P P - - Q C R C T D V R L N
5	- V K S A	C C D T C	L C T R S Q	- - P P - - T C R C V D V G E R
6	- S S E P	C C D S C	R C T K S I	- - P P - - Q C H C A D I R L N
7	- V K S A	C C D T C	L C T K S E	- - P P - - T C R C V D V G E R
8	T T A	C C N F C	P C T R S I	- - P P - - Q C R C T D I G E T
9	R P W K -	C C D N I	K R L P T K P D P P	- - Q W R C N D E L E P
9*	R P W G D	C C D K A F	C N K M N	- - P P - - T C R C M D E V K -
10	R P W K -	C C D R A I	C T K S F	- - P P - - M C R C M D M V E -
11	R P W K -	C C D Q A V	C T R S I	- - P P - - I C R C M D Q V F E
12	- V K S A	C C D T C	L C T K S E	- - P P - - T C R C V D V G E R
13	R P W E -	C C D I A M	C T R S I	- - P P - - I C R C V D K V D R

↓

40

50

60

1	-	S	C	H	S	A	C	D	R	C	A	C	T	R	S	M	-	-	P	-	-	-	G	C	R	C	L	D	T	T	D	F	
2	-	-	C	P	A	S	C	N	S	C	V	C	T	R	S	N	-	-	P	P	-	-	Q	C	R	C	T	D	K	T	Q	C	
3	-	S	C	H	S	A	C	K	S	C	A	C	T	Y	S	I	-	-	P	A	-	-	K	C	F	C	T	D	I	N	D	F	
4	-	S	C	H	S	A	C	S	S	C	V	C	T	F	S	I	-	-	P	A	-	-	Q	C	V	C	V	D	M	K	D	F	
5	-	-	C	H	S	A	C	N	H	C	V	C	N	Y	S	N	-	-	P	P	-	-	Q	C	Q	C	F	D	T	H	K	F	
6	-	S	C	H	S	A	C	K	S	C	M	C	T	R	S	M	-	-	P	G	-	-	K	C	R	C	L	D	T	D	F	F	
7	-	-	C	H	S	A	C	N	S	C	V	C	R	Y	S	N	-	-	P	P	-	-	K	C	Q	C	F	D	T	H	K	F	
8	-	-	C	H	S	A	C	K	T	C	L	C	T	K	S	I	-	-	P	P	-	-	Q	C	R	C	T	D	I	T	N	F	
9	S	Q	C	T	A	A	C	K	S	C	R	E	A	P	G	P	F	G	K	L	I	-	-	C	F	D	I	Y	W	G	A	D	
9*	-	E	C	A	D	A	C	K	D	C	Q	R	V	E	S	S	E	-	P	P	R	Y	V	C	K	D	R	F	T	G	H	P	
10	-	Q	C	A	A	T	C	K	K	C	G	P	A	T	S	D	S	S	R	R	-	-	V	C	E	D	X	Y					
11	-	-	C	P	S	T	C	K	A	C	G	P	S	V	G	D	P	S	R	R	-	-	V	C	Q	D	Q	Y	V				
12	-	-	C	H	S	A	C	N	S	C	V	C	R	Y	S	N	-	-	P	P	-	-	K	C	Q	C	F	D	T	H	K	I	
13	-	-	C	S	D	A	C	K	D	C	E	E	T	E	D	-	-	N	R	H	-	-	V	C	F	D	T	Y	I	G	D	P	

40

50

70

1	-	C	Y	K	P	-	C	K	S	S	D	E	D	D	D			
2	R	C	P	V	T	E	C	R	S									
3	-	C	Y	E	P	-	C	K	S	S	R	D	D	D	W	D	N	
4	-	S	Y	A	P	-	C	K	S	S	H	D	D					
5	-	C	Y	K	A	-	C	H	S	S	E	K	E	E	V	I	K	N
6	-	C	Y	K	P	-	C	E	S	M	D	K	D	D	D			
7	-	C	Y	K	S	-	C	H	N									
8	-	C	Y	P	K	-	C	N										
9	P	-	-	G	P	F	C	T	P									
9*	-	-	-	G	P	V	C	K	P	R								
12	P	A	P	M	-	-	C	H	E	H	Q							
13	-	-	-	G	P	T	C	H	D	D								

60

Ikenaha, 1983; Yoshikawa et al., 1979; Joubert et al., 1979; Shimokawa et al., 1984; Wilson and Chen, 1983; Brow et al., 1985; Asao et al., 1991).

The legume inhibitors have been shown to be "double-headed", with one inhibitory site in each of two structural domains. Similar domains are present in the Coix inhibitor, where they correspond to residues 1-31 and 32-64, respectively. The Coix inhibitor TII has greatest homology with the cereal proteins (48-55% identity of residues), but there is also significant homology with the legume Bowman-Birk inhibitors (27-37% identity). Ten out of the 14 cysteine residues which are characteristic of the Bowman-Birk family are conserved in the Coix inhibitor, and the sequence identity is particularly high in the region around the first reactive site of the legume inhibitors (in domain I). This homology suggests that the trypsin reactive site in the Coix TII is Arg(17)-Ser(18) as this peptide bond is located in an homologous position to the first reactive site of the legume inhibitors, and moreover obeys the tentative rules for such sites (Laskowski and Kato, 1980). There is no such identifiable peptide bond in the region of the Coix sequence which corresponds with the location of the second reactive site in domain II of the double-headed legume inhibitors. It is probable, therefore, that the Coix protein is a single-headed inhibitor similar to the type II inhibitors in wheat germ (Odani et al., 1986) and foxtail millet (Tashiro

et al., 1990), and is not double-headed as suggested by Ohtsubo et al. (1985) who based their calculations of the stoichiometry of inhibition on an incorrect estimate of the molecular mass of the inhibitor protein. The larger type I inhibitor of rice bran (Tashiro et al., 1987) differs in the presence of an internal duplication, residues 70-133 (in Fig. 7) corresponding to residues 3-69 (in Fig. 7). The sequences reported for the type I and II inhibitors of wheat are incomplete, but an internal duplication also appears to be present in the former (Odani et al., 1986).

Using the FASTP and RDF computer programmes (Lipman and Pearson, 1985), limited homology of low significance can also be detected between a short region of the Coix inhibitor (residues 9-25) and sections of the sequences of the trypsin inhibitor from rye (residues 44-57) (Lyons et al., 1987) and barley (residues 43-56) (Odani et al., 1983a), which are classified in the cereal superfamily. Such weak affinities between the Bowman-Birk and cereal families have been noted previously (Kreis et al., 1985).

### 3.3 Prediction of Hydropathy and Secondary Structure

Fig. 8 shows a comparison of the hydropathy profiles of the Coix trypsin inhibitor, wheat germ type II inhibitor and soybean Bowman-Birk CII inhibitor. As might be expected the two cereal inhibitors show the greatest similarities, but all three proteins are similar in possessing hydrophilic regions near their N- and C- termini, and more hydrophobic regions around the putative reactive sites. Also shown in Fig. 8 are the secondary structures predicted from the amino acid sequences. It can be seen that the only regions showing much similarity are those around the reactive sites. It should be noted, however, that the Coix and wheat inhibitors have similar predicted contents of  $\alpha$ -helix (34 and 37 %, respectively) and  $\beta$ -turn (55 and 52 %, respectively), whereas the soybean CII has a higher content of  $\beta$ -turn (81%) and no  $\alpha$ -helix. Such high levels of  $\beta$ -turns and the frequency of disulphide bridges may account for the unusual thermal stability of these proteins.

### PURIFICATION, CHARACTERIZATION AND SEQUENCING OF AN INSECT $\alpha$ -AMYLASE INHIBITOR/ENDOCHITINASE FROM SEEDS OF JOB'S TEARS (Coix lachryma-jobi)

#### 3.4 Properties of Locust Gut $\alpha$ -Amylase

The crude enzyme preparations (see section 2.13) from locust gut catalysed the hydrolysis of starch, with a pH optimum

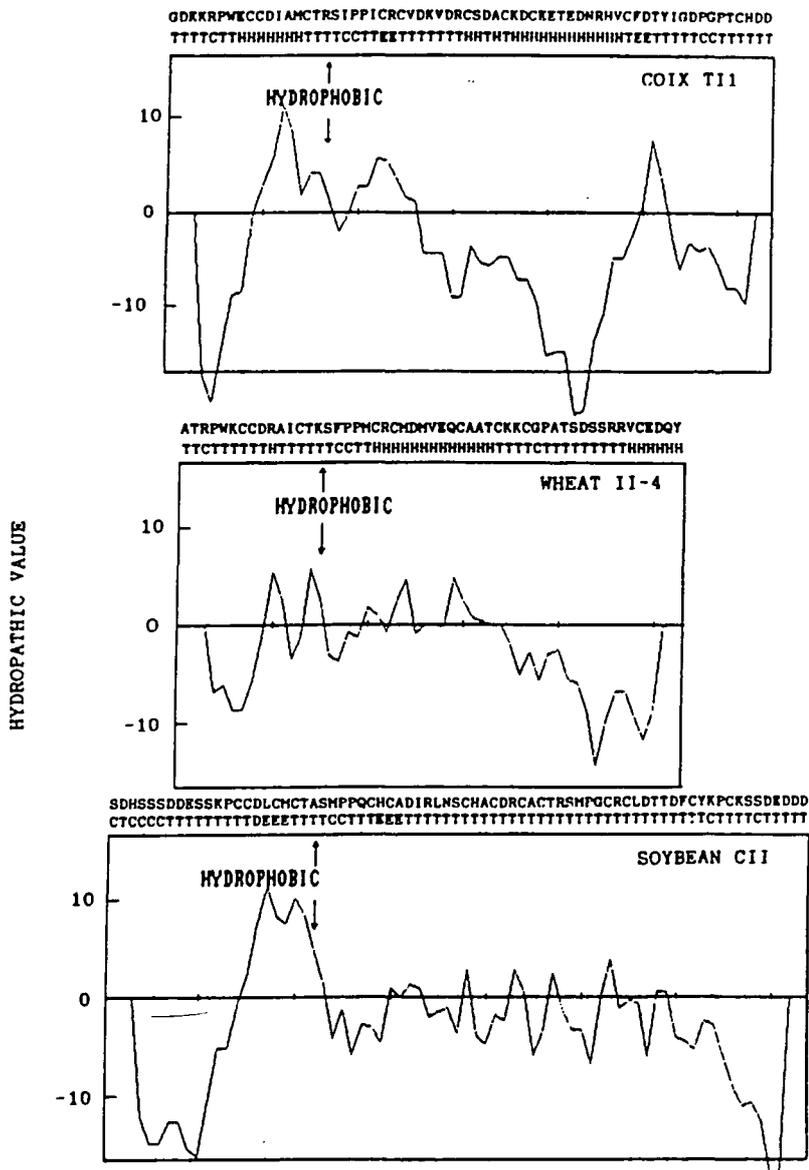


Figure 8. Comparison of the hydropathy profiles and secondary structures predicted from the amino acid sequences of the Coix TII (top), wheat germ II-4 (middle; Odani et al., 1986) and soybean CII (bottom; Odani and Ikenaka, 1977). The Kyte and Doolittle (1982) plots of hydropathy were computed as described in Krystek et al. (1985). The secondary structures were predicted using the computer method of Garnier et al. (1978). H,  $\alpha$ -helix; E,  $\beta$ -sheet; T,  $\beta$ -turn and C, random coil. Arrows indicate the positions of the putative reactive sites.

of about 7.0 (Fig. 9). The reaction was linear with 2.5 to 20  $\mu$ l of enzyme preparation per assay (Fig. 10), but became non-linear at higher volumes. A volume of 15  $\mu$ l was therefore used for all assays.

### 3.5 Purification of the Coix $\alpha$ -Amylase Inhibitor

The  $\alpha$ -amylase inhibitor was extracted from Coix seeds as described in section 2.5. The amylolytic activity of the locust enzyme preparation was strongly inhibited by the neutralised protein extract from Coix seeds, and by the two fractions precipitated between 0- 30% and 30- 60% with ammonium sulphate (Fig. 11). No inhibition occurred with the fractions precipitated between 60 and 90% ammonium sulphate (data not shown).

The proteins precipitated between 0 and 60% ammonium sulphate were bulked and applied to a column of Red Sepharose CL-6B. This is an affinity absorbant with a procion red HE-3B ligand. Although marketed as an absorbant for purifying NADP-dependent enzymes and carboxypeptidase G, it has previously been shown to bind some plant inhibitors of  $\alpha$ -amylase (Kutty, 1988). The molecular basis for this binding is not known. Inhibitory activity was present in the unbound fractions and in the fraction eluted with 3.0 M NaCl (Fig. 12). However, when the unbound fraction was re-applied to a second column all the inhibitory activity was bound, and could then be eluted as

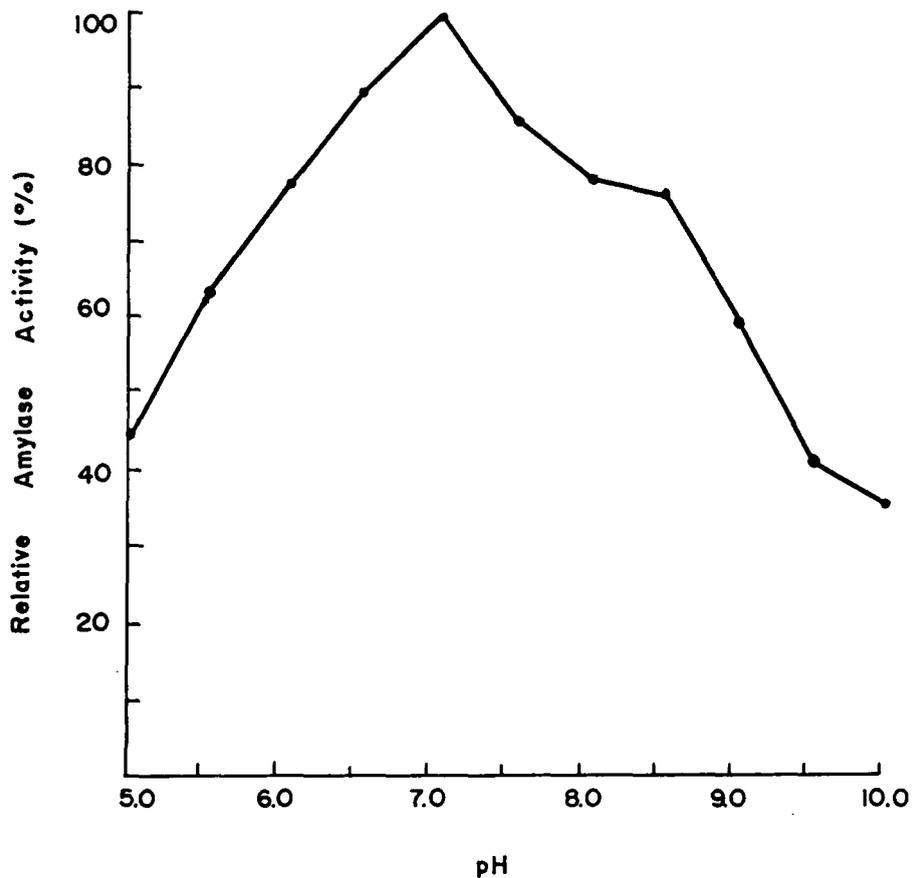


Figure 9. Effect of pH on hydrolysis of soluble starch at 30°C by gut homogenates of larvae of locusts (Locusta migratoria migratorioides). The following buffers were used at 50 mM concentrations: acetate, (pH 5.0-5.5), phosphate (pH 6.0-7.0) and Tris-HCl (pH 7.5-10). Relative activities were determined from duplicate analyses.

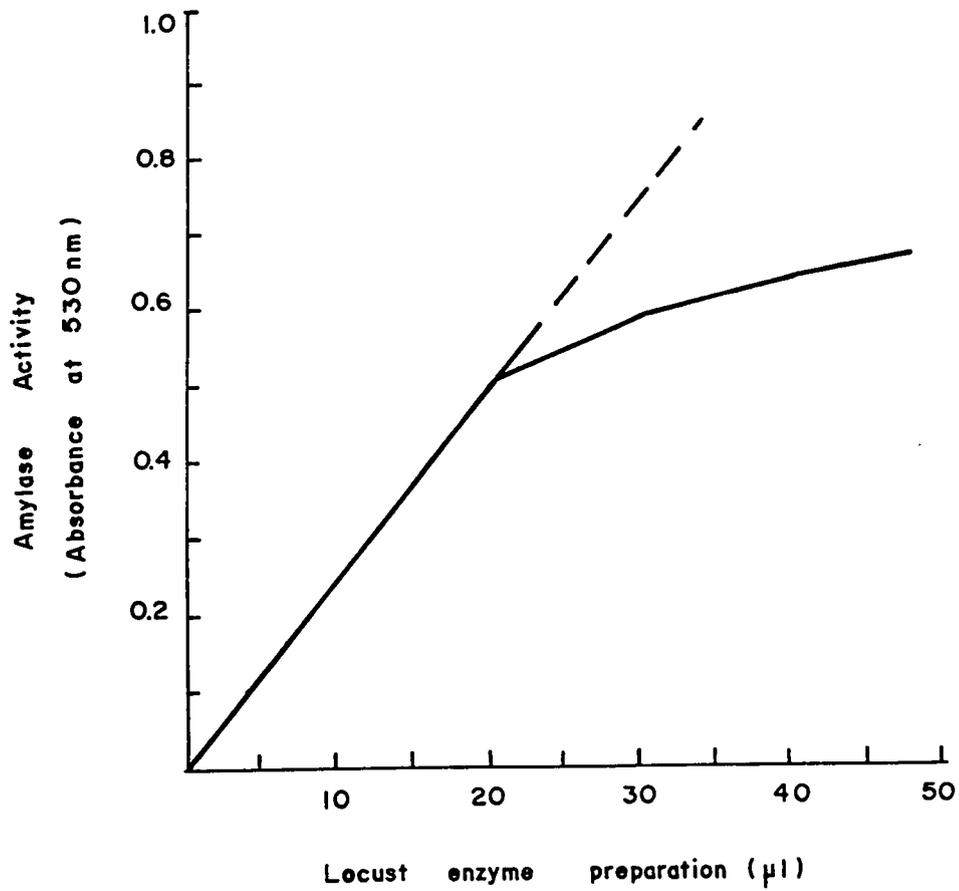


Figure 10. Starch hydrolyzing activity of the crude enzyme preparation from locust gut. The amylase activity was measured as described in section 2.7 using 0.05M sodium acetate buffer (pH 7.0).

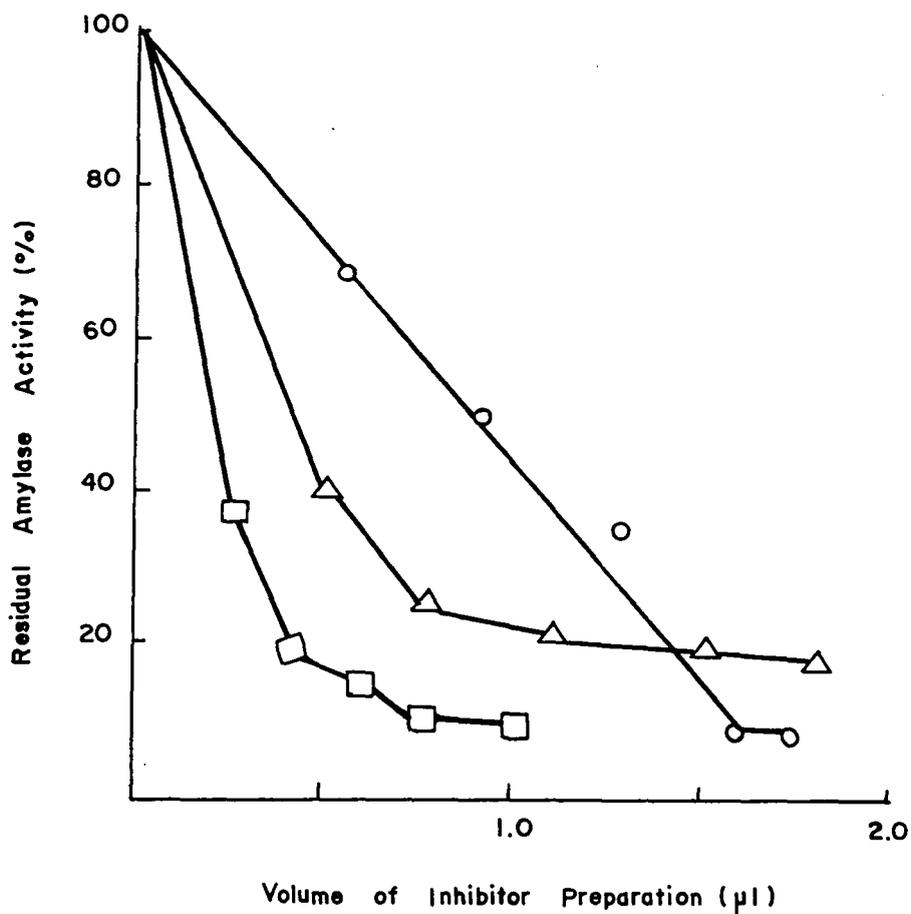


Figure 11. Inhibition of the locust gut amylase by the neutralised protein extract from *Coix* seeds ( O ) and by two fractions precipitated between 0-30% ( Δ ) and 30-60% ( □ ) ammonium sulphate. Increasing volumes of each sample were mixed with the same volume of the locust gut preparation. The residual amylase activity was measured as described in section 2.7.

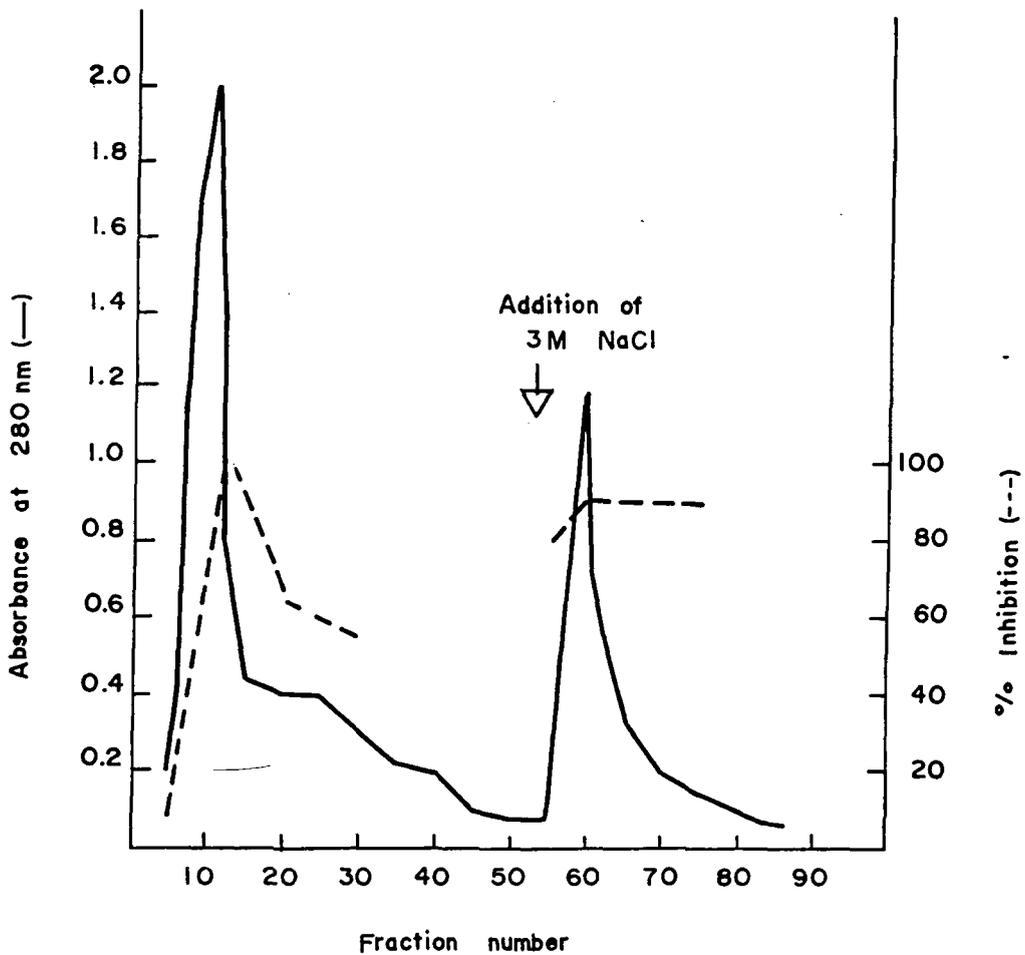


Figure 12. Separation of the 60% ammonium sulphate fraction on a column (8.5 x 3.0 cm) of Red Sepharose CL-6B. The protein was applied to the column in 0.05M Tris-HCl buffer containing 0.1M NaCl. After removal of unbound protein with the same buffer the bound material was eluted by the addition of 3M NaCl. Fractions (10 ml) containing unbound and bound material were tested for inhibition of locust amylase.

before (Fig. 13). The bound protein from the two columns was then bulked and separated by HPLC, using a preparative C18 column (Fig. 14). Two major peaks were eluted, at acetonitrile concentrations of 46 and 47% (called peaks 1 and 2, respectively, in Fig. 14). Both peaks inhibited the amylolytic activity of the locust gut preparations, the total yield being about 1.0 mg per 400 g flour ( 0.007%). The first peak (at 46% acetonitrile) accounted for about 75% of the total.

### 3.6 Inhibitory Properties

Fractions from the purification stages (the neutralised acidic extract, ammonium sulphate fractions and Red Sepharose fractions) were tested for inhibition of  $\alpha$ -amylases from Bacillus subtilis, porcine pancreas and human saliva. No inhibition was observed even when the inhibitor fractions were pre-incubated with the enzyme for 3 h at 37°C in 0.1 M acetate buffer (pH 7.0) containing 20 mM NaCl and 100 mM CaCl<sub>2</sub>. The addition of bovine serum albumin (0.4 mg/ml) also had no effect.

The purified inhibitor (46% acetonitrile HPLC peak) was re-tested against these  $\alpha$ -amylases and also against enzymes from Aspergillus oryzae and barley malt. The enzymes and purified inhibitor were mixed at molar ratios of 1:1 and allowed to stand for 25 min at 37°C before assay. No inhibitory activity was observed. These results are not typical of cereal  $\alpha$ -amylase

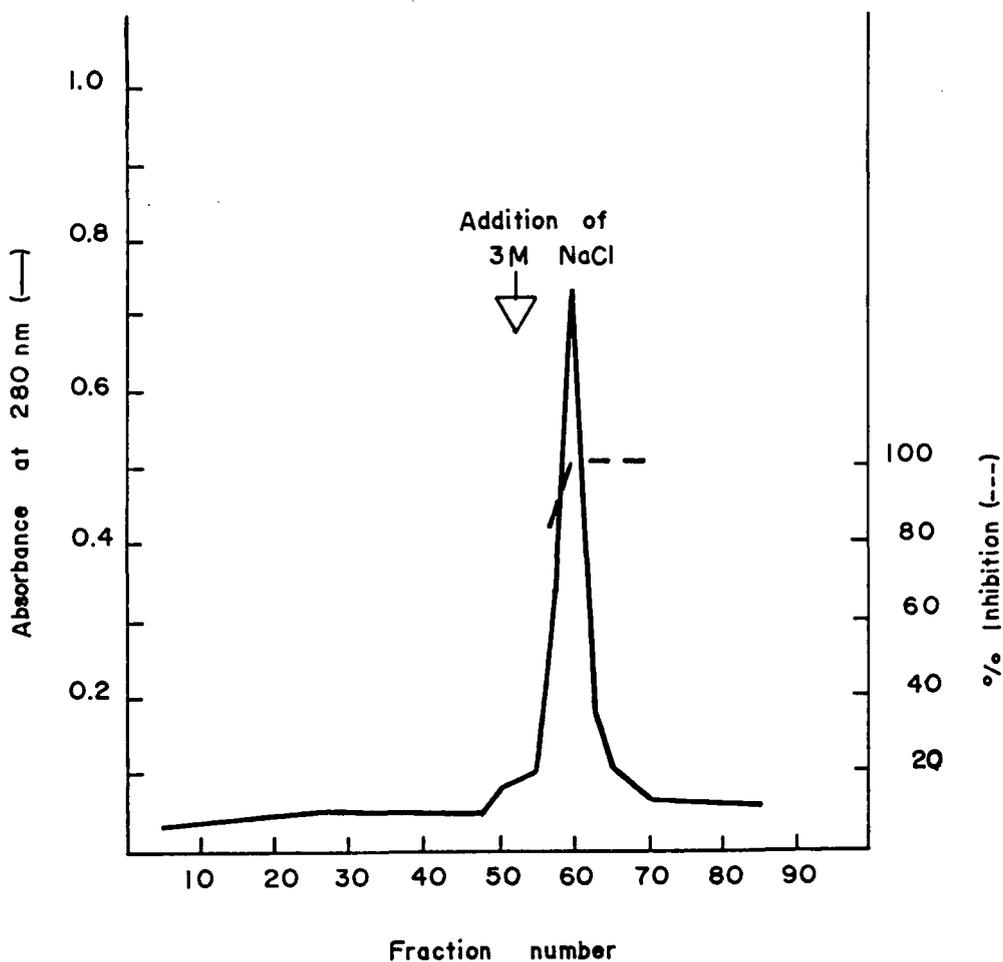


Figure 13. Rechromatography of the unbound fraction from Red Sepharose CL-6B on a second column (8.5 x 3.0 cm) of Red Sepharose CL-6B. The unbound fraction was applied to the column in 0.05M Tris-HCl buffer containing 0.1M NaCl. The bound material was eluted by the addition of 3M NaCl and tested for inhibition of locust amylase.



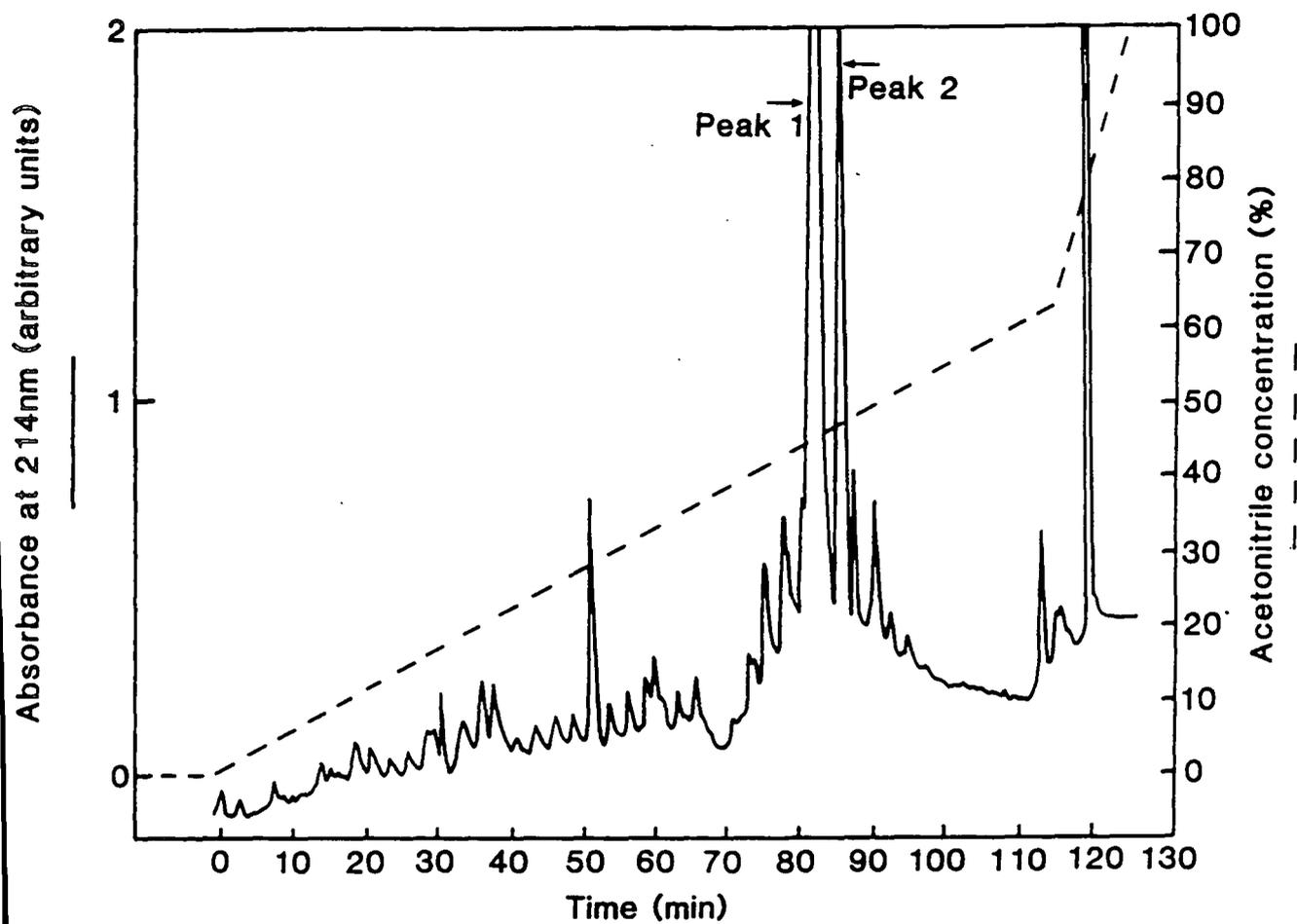


Figure 14. Reversed-phase HPLC of the bound fraction from the Red Sepharose CL-6B column on a preparative C18 column (25 cm x 12 mm, Vydac 218TP1022), equilibrated in 0.1% trifluoroacetic acid and eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 10ml / min.

inhibitors since many inhibitors of heterologous  $\alpha$ -amylase were isolated from cereal seeds (Garcia-Olmedo et al., 1987; Bloch Jr. and Richardson, 1991).

### 3.7 Characterization of the Insect $\alpha$ -Amylase Inhibitor/ Endochitinase from Coix Seeds

SDS-PAGE analysis of the two major peaks from the HPLC separation gave identical patterns. In each case a major band of  $M_r$  about 52 500 was observed under non-reducing conditions, which was replaced by a lower  $M_r$  band in the presence of 2-mercaptoethanol (Fig. 15). The  $M_r$  of this latter band was determined using reduced and carboxymethylated material from the first (46% acetonitrile) peak as 26 400. Isoelectric focusing of the two fractions under denaturing and reducing conditions showed similar patterns, with a diffuse band with a pI value between about 8.5 and 9.0 (Fig. 16). Their amino acid compositions (Table VI) were also similar, with high contents of aspartate/asparagine, glutamate/glutamine, glycine and alanine. These results indicate that the proteins in the two major HPLC peaks are closely related. In each case the native protein appears to be a dimer, consisting of two identical or closely similar subunits. The conversion of the dimer to monomer in the presence of a reducing agent indicates that the dimers are stabilized by disulphide bonds. Particularly in the case of the

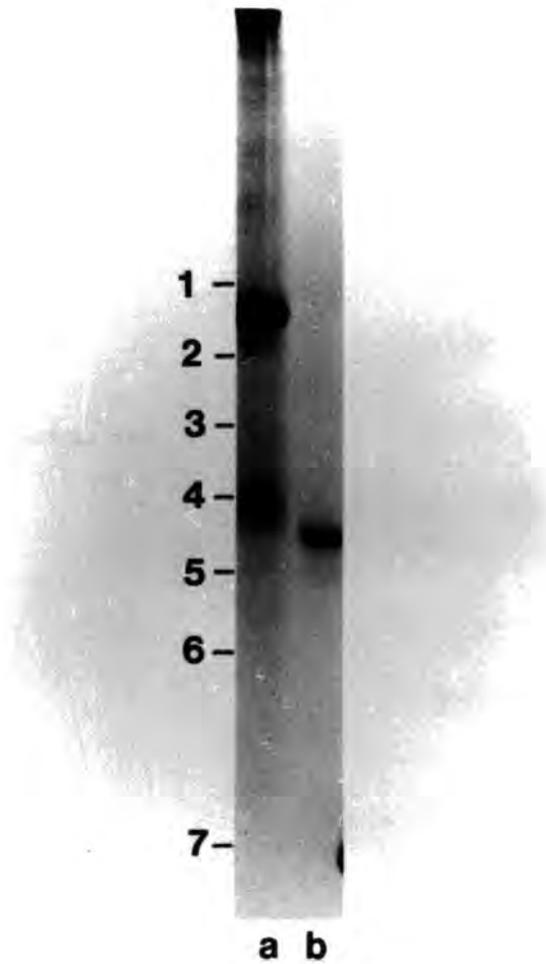


Figure 15. SDS-PAGE of the peak eluted from the reversed-phase HPLC column at 46% acetonitrile in the native state (track a) and after reduction and carboxymethylation (track b). The numbers indicate the position of marker proteins of known molecular weight. These are: 1, bovine serum albumin (66 000); 2, egg albumin (45 000); 3, glyceraldehyde-3-phosphate dehydrogenase (36 000); 4, carbonic anhydrase (29 000); 5, trypsinogen (24 000); 6, soybean trypsin inhibitor (20 000); 7,  $\alpha$ -lactalbumin (14 200).

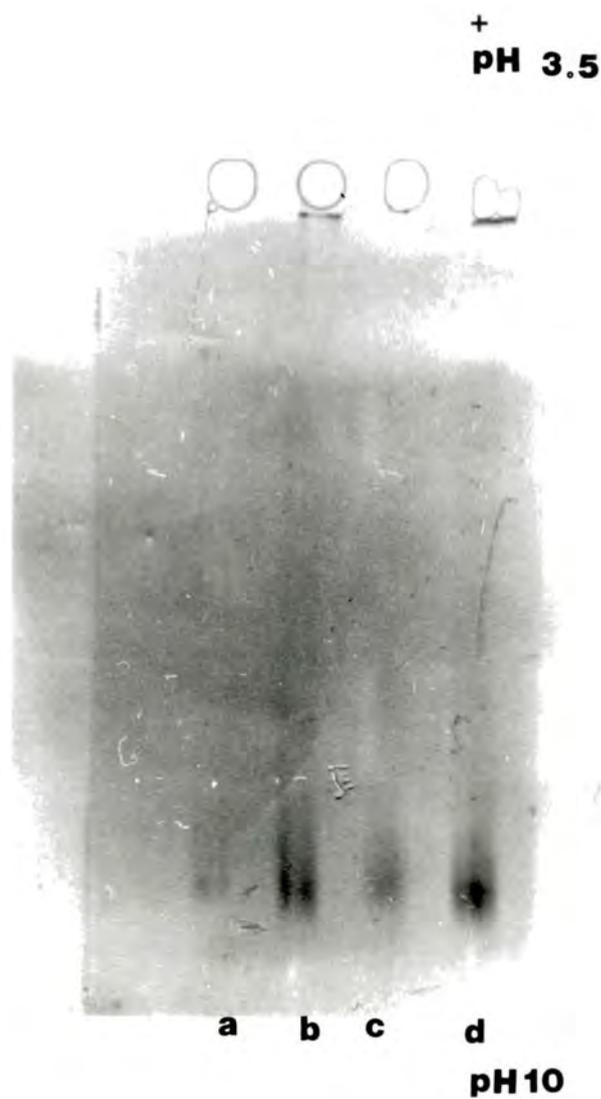


Figure 16. Isoelectric focusing of the Coix peaks eluted at 46 and 47% (v/v) acetonitrile. The letters indicate the peak acetonitrile concentration and volume in each track. These are: track a (46% peak; 10 $\mu$ l); track b (46% peak; 20 $\mu$ l); track c (47% peak; 10 $\mu$ l) track d (46% peak; 20 $\mu$ l).

TABLE VI - Comparison of the amino acid compositions (expressed as mol%) of the Coix peaks eluted at 46 and 47% (v/v) acetonitrile with those reported for basic endochitinases from barley, wheat, bean, tobacco and potato (Leah et al., 1987; Molano et al., 1979; Broglie et al., 1986; Shinski et al., 1987; Gaynor, 1988).

Amino acid	Coix		Barley	Wheat	Bean	Tobacco	Potato
	46%	47%					
Asx <sup>a</sup>	12.9	12.7	9.1	9.4	9.7	11.3	13.2
Thr	5.3	5.6	5.4	7.4	7.3	4.9	4.0
Ser	6.0	6.3	6.9	8.1	8.7	8.3	7.6
Glx <sup>b</sup>	9.5	10.1	7.0	6.7	7.3	6.3	6.0
Pro	2.6	2.5	6.6	5.1	6.7	7.3	7.3
Gly	15.5	13.4	12.0	17.5	12.3	14.9	14.6
Ala	11.7	11.2	14.5	9.1	8.7	7.0	7.0
Cys	2.7	2.7	2.8	4.0	5.3	5.7	5.6
Val	4.7	5.1	4.9	4.7	3.3	2.7	3.0
Met	1.0	1.1	0.6	1.0	0.7	1.3	1.0
Ile	3.3	3.5	4.2	3.0	3.7	5.0	5.0
Leu	3.3	3.7	4.4	4.4	5.7	4.7	4.6
Tyr	5.6	5.8	4.6	4.7	5.0	3.7	3.6
The	5.4	5.3	4.9	4.7	4.3	4.7	5.0
His	1.8	1.9	1.9	1.3	1.0	1.3	1.3
Lys	5.1	5.1	2.7	2.7	2.7	2.3	2.6
Arg	3.6	4.1	5.9	4.7	5.3	6.0	6.0
Trp	n.d	n.d	1.4	1.3	2.3	2.7	2.6

<sup>a</sup> Asp/Asn

<sup>b</sup> Glu/Gln

n.d., not determined

46% acetonitrile peak the two subunits should be identical since no evidence of sequence heterogeneity was observed during the determination of its amino acid sequence

### 3.8 Determination of the Partial Amino Acid Sequence

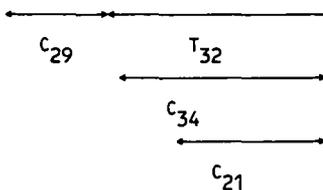
Determination of the partial amino acid sequence was carried out on the 46% acetonitrile HPLC peak, by using the manual DABITC/PITC method. The total number of residues sequenced in this way was about 130, equivalent to almost half of the total number expected from the  $M_r$  of the protein subunit. No heterogeneity in the sequence was observed.

Comparison of the peptide sequences with the published amino acid sequences of other proteins showed no similarities with any other known  $\alpha$ -amylase inhibitors or enzyme inhibitors, but did show unexpected homologies with partial and complete amino acid sequence of endochitinases from a number of sources including barley seeds, embryogenic barley suspension cultures and leaves of tobacco, potato and bean. In fact, almost all of the tryptic and chymotryptic peptides could be aligned with these sequences including a continuous region of about 70 residues (Fig. 17). None of the peptides corresponded to the N-termini of the other proteins, and attempts to determine the N-

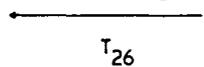
Figure 17. Alignment of the amino acid sequences of the tryptic and chymotryptic peptides of the Coix  $\alpha$ -amylase inhibitor/endochitinase with those reported for basic endochitinases from barley seeds (Leah et al., 1987), barley cultures (Kragh et al., 1991), bean (Broglie et al., 1986) and potato (Gaynor, 1988) and for basic (Shinski et al., 1987) and acidic endochitinase (van Huijsduijnen et al., 1987) from tobacco. The sequences are aligned to maximise homology, resulting in the introduction of some gaps. Standard single letter abbreviations are used. Individual Coix peptides are shown by arrows and numbered.

RESIDUE NUMBER

1 50  
 Barley (cultures) EQ - G S Q A G G A T C P N X L C C S R F G  
 Bean EQ C G R Q A G G A L C P G G N C C S Q F G W C G S T T D Y C - P G - C Q S Q C - - G G P S P A P T  
 Potato Q N C G S Q G G G K A C A S G Q C C S K F G W C G N T N D Y C G S G N C Q S Q C P G G G P G P G  
 Tobacco (basic) E Q C G S Q A G G A R C A S G L C C S K F G W C G N T N D Y C G P G N C Q S Q C P G G - P T P P G C  
 Coix C C S K F G Y C G L T D A Y



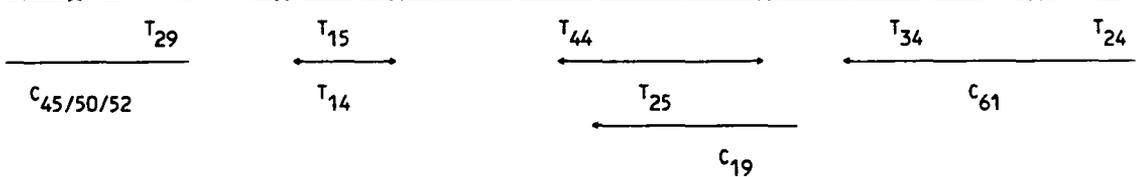
51 100  
 Barley (seeds) - S V S S I V S R A Q F D R M L L H R N D G A T Q A K G F Y T Y D A F V A A A A A F P G F G R T G S  
 Bean - D L S A L I S R S T F D Q M L K H R N D G A C P A K G F Y T Y D A F I A A A K A Y P S F G N T G D  
 Potato G D L G S A I S N S M I D Q M L K H R N L N S C Q G K N F Y S Y N A F I N A A R S F P G F G T S G C  
 Tobacco (basic) G D L G S I I S S S M F D Q M L K H R N D N A C Q G K G F Y S Y N A F I N A A R S F P G F G T S G C  
 Coix N F Y T G Q L I T S



101 150  
 Barley (seeds) A D A R K  
 Bean T A T R K R E I A A F L G Q T S H E T T G G W A T A P D G P Y A W G Y C - F Y R E R N P S T Y C -  
 Potato I N A R K R E I A A F F A G T S H L T T G G W A S A P D G P Y A W G Y C - F L R E R G N P G D Y C -  
 Tobacco (basic) T T A R K R E I A A F F A Q T S H E T T G G W A T A P D G P Y A W G Y C W L - R E Q G S P G D Y C T  
 Coix F A H V T H E T G N N A Y C D



151 200  
 Barley (cultures) G P I Q I S H N Y N Y G P - A G R  
 Bean S A T P Q F P C A P G Q Q Y Y G P G P I Q I S W N Y N Y G Q C G R A I G V D L L N K P D L V A T D S  
 Potato P P S S Q W P C A P G R K Y F G P G P I Q I S H N Y N Y G P C G R A I G V D L L N N P D L V A T D P  
 Tobacco (basic) P S - G Q W P C A P G R K Y F G R G P I Q I S H N Y N Y G P C G R A I G V D L L N N P D L V A T D P  
 Tobacco (acidic) V A T D P  
 Coix P S K T Q K P C A A G K K Y Y G R G P I Q I S X N Y N Y G P A G R A I G M D G L G N P D R V A Q D A



201

250

Barley (cultures)	F W M T P Q S P K P S - - D V I T G N	
Barley (seeds)	M T A Q P P K P S S H A V I A G Q W S P D G A D R A A G R V P G F G V I T N I	
Bean	V I S F K S A L W F W M T A Q S P K P S S H D V I T S R W T P S S A D V A A R R L P G Y G T V T N I	
Potato	V I S F K T A L W - - M T P O S P K P S C H D V I I G R W N P S S A D R A A N R L P G F G V I T N I	
Tobacco (basic)	V I S - K S A L W F W M T P Q S P K P S C H D V I I G R W Q P S S A D R A A N R L P G F G V I T N I	
Tobacco (acidic)	V I S F K S A L W F W M T P Q S P K P S C H D V I I G R W Q P S S A D R A A N R L P G F G V I T N I	
Coix	<sup>L</sup> I D D Y K T A L X F <sup>L</sup> I V N G E E A V P G	L S A A N A

251

300

Barley (cultures)	Y C D L L G V S Y G D N L D C Y N Q R P F A	
Barley (seeds)	I N	
Bean	I N G G L E C G R G Q D S R V Q D R I G F F K R Y C D L L G V G Y G N N L D C Y S Q T P F G N S L L	
Potato	I N G G L E C G R G T D N R V Q D R I G F Y R R Y C S I L G V T P G D N L D C V N Q R W F G N A L L	
Tobacco (basic)	I N G G L E C G R G T D S R V Q D R I G F Y R R Y C S I L G V S P G D N L D C G N Q R S F G N G L L	
Tobacco (acidic)	I N G G L E C G R G T D S R V Q D R I G F Y R R Y C S I L G V S P G D N L D C G N Q R S F G N G L L	
Coix	V S Y Y R Q Y C Q Q L G V D P G P N L	

301

Bean	L S D L V T S Q
Potato	V D T L
Tobacco (basic)	V D T M
Tobacco (acidic)	V D T M

terminal sequence of the intact Coix protein using automated solid-phase and gas-phase sequencers showed that it was blocked.

Although all of the complete amino acid sequences shown in Fig. 17 correspond to basic endochitinases, Jamet and Fritig (1986) have shown that the acidic endochitinases of tobacco leaves are serologically related to the basic type, while van Huijsduijnen et al. (1987) have shown that partial amino acid sequences of the two types are about 70% homologous to each other and to the bean endochitinase. Samac et al. (1990) have cloned and determined the nucleotide sequence of the genes encoding the acidic and basic chitinases from Arabidopsis thaliana. The basic chitinase had 73% amino acid sequence similarity to the basic chitinase from tobacco, and the acidic chitinase has 60% amino acid sequence similarity to the acidic chitinase from cucumber (Metraux et al., 1989). However, the Arabidopsis acidic and basic chitinases are not related. It is of interest that the N-termini of the bean and potato enzymes are strongly homologous with the N-termini of wheat germ lectin (Lucas et al., 1985). Recently, Araki et al. (1992) have shown a homology of 52% between the N-terminal domain of an acidic chitinase from yam (Dioscorea japonica) tuber and wheat germ lectin. Our identification of a peptide with the characteristic Cys-Cys-Ser sequence

indicates that at least part of this "lectin-like" sequence is present in the Coix protein. It is also of interest that the carboxyl-terminal domain of a cDNA clone encoding a lectin shown unexpected similarities with the amino acid sequences of chitinases from potato, tobacco (basic and acidic ones), bean and barley (Lerner and Raikhel, 1992)

The two proteins purified from Coix (46 and 47% acetonitrile) also had similar amino acid compositions to basic endochitinases from other sources (see Table VI), most notably high contents of glycine (12-17 mol%) and fairly high contents of aspartate/asparagine (9-13 mol%) and alanine (7-14 mol%).

### 3.9 Endochitinase Activity

Because of sequence homology of the Coix  $\alpha$ -amylase inhibitor with endochitinases from other sources this inhibitor (46% acetonitrile peak) was assayed for endochitinase activity.

Incubation of the 46% acetonitrile HPLC peak with tritiated chitin resulted in the release of TCA-soluble radioactivity, demonstrating endochitinase activity. 1.0 mg of protein released about 5 nmol of N-[<sup>3</sup>H] acetylglucosamine during the 15 min assay period. This activity is lower than those reported for enzymes from wheat (Molano et al., 1979) and barley (Leah et al., 1987), which may be due to partial denaturation during the lengthy purification procedure. The

identification of a protein with this combination of functions, as an enzyme inhibitor (see section 3.5) and as enzyme is the most unexpected result of this study.

#### MOLECULAR METHODS - ISOLATION AND CHARACTERIZATION OF mRNA FRACTIONS

All the molecular methods carried out in this work (see section 2.18 to section 2.24) were done with the objective of preparing a cDNA library in order to isolate and characterise corresponding cDNA clones to the Coix  $\alpha$ -amylase inhibitor/endochitinase. Unfortunately, due to lack of time the study could not be completed. Some results obtained during these studies are described below.

##### 3.10 Antigenic Relationships of the Coix $\alpha$ -amylase Inhibitor/Endochitinase

Polyclonal antibodies were raised against the purified Coix  $\alpha$ -amylase/endochitinase (46% acetonitrile peak), as

described in section 2.18. Using the immuno-assay method described in section 2.19, the antigenic relationships of the Coix protein were studied using this antiserum and two antisera for sugar beet and wheat germ chitinases (kindly supplied by Dr. Jørn Mikkelsen).

The  $\alpha$ -amylase inhibitor/endochitinase from Coix reacted strongly with the Coix, sugar beet and wheat germ antisera (Fig. 18). Only a weak reaction was observed between the Coix protein and a non-immune antiserum (Fig. 18).

### 3.11 Isolation and Characterization of mRNA from Developing Seeds of Coix

Poly (A<sup>+</sup>)-rich mRNA was isolated from whole Coix seeds between 20 and 30 days after anthesis (see section 2.20). The purity of the RNA preparation was estimated by measuring the absorbance at 260 and 280 nm (Maniatis et al., 1982), showing a high level of purity ( $A_{260nm}/A_{280nm}=1.9$ ). Approximately 200  $\mu$ g of poly (A<sup>+</sup>) mRNA was obtained from 50 g of whole Coix seeds.

#### 3.11.1 In vitro Translation and Immunoprecipitation

Poly (A<sup>+</sup>) -rich mRNA was translated using wheat germ cell-free extracts supplemented with [<sup>3</sup>H] leucine as described by Kreis et al. (1983). The products of in vitro translation

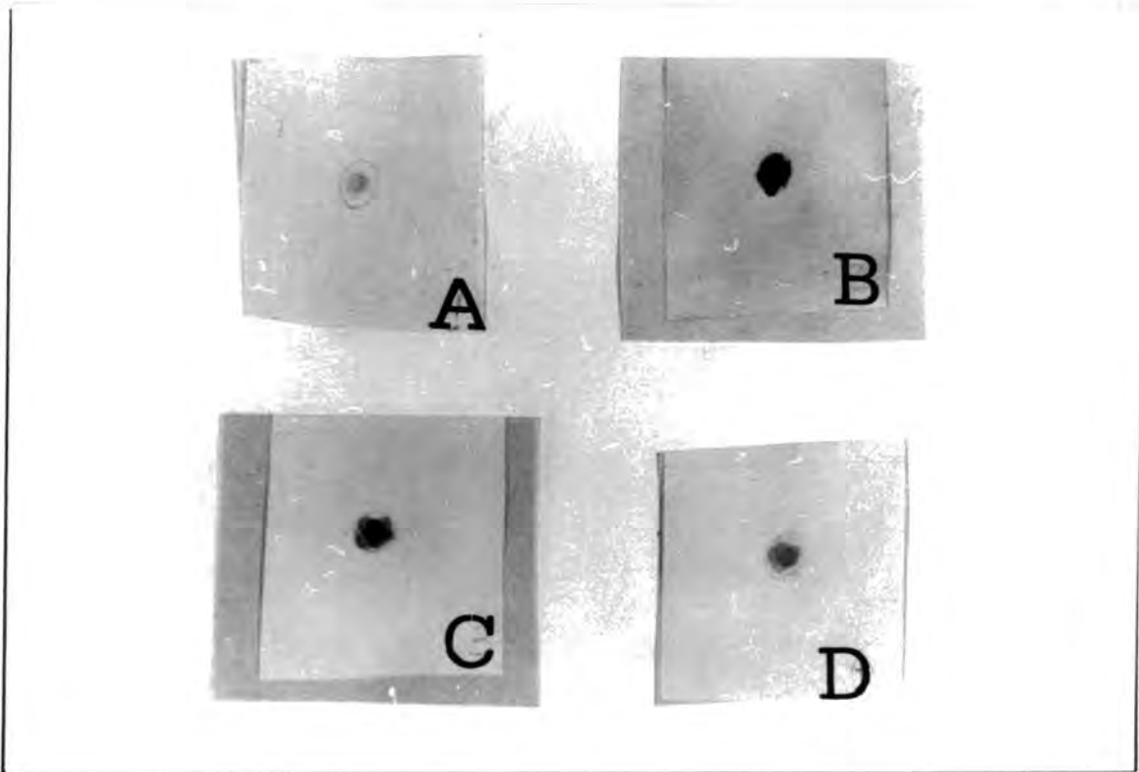


Figure 18. Immunoassay of the  $\alpha$ -amylase inhibitor/endochitinase from Coix seeds (see section 2.19)

A) non - immune antiserum

B) antiserum raised against the Coix  $\alpha$ -amylase inhibitor/endochitinase

C) antiserum raised against the sugar beet endochitinase

D) antiserum raised against the wheat germ endochitinase

were clearly visualized by SDS-PAGE and autoradiography (Fig. 19), indicating that the mRNA fraction was of good biological quality.

The translation products were immunoprecipitated using the Coix  $\alpha$ -amylase inhibitor/endochitinase antiserum, sugar beet endochitinase antiserum and a non-immune antiserum, and then subjected to SDS-PAGE. Clear single bands were precipitated with all the antibodies, although the band precipitated by the non-immune antiserum was very weak (Fig. 20). These bands correspond to the strongest band visualized by SDS-PAGE of the translation products, with a  $M_r$  of about 25k (see Fig. 19).

### 3.12 Characterization of a Bean Endochitinase cDNA

A bean (Phaseolus vulgaris) cDNA, cloned into the EcoR1 site of pUC13, (pCH18 kindly supplied by Dr. Jack Gaynor, of the State University of New Jersey, Newark, New Jersey) was cleaved with EcoR1 and was run on agarose gel electrophoresis under conditions as described in section 2.24 to check that it contained the correct insert. The result was positive since it gave two bands on agarose gel electrophoresis (Fig. 21). The major band of ca. 2.8 kb corresponds to the linearized vector and the minor band of ca. 1.1 kb corresponds to the insert as reported by Broglie et al. (1986).

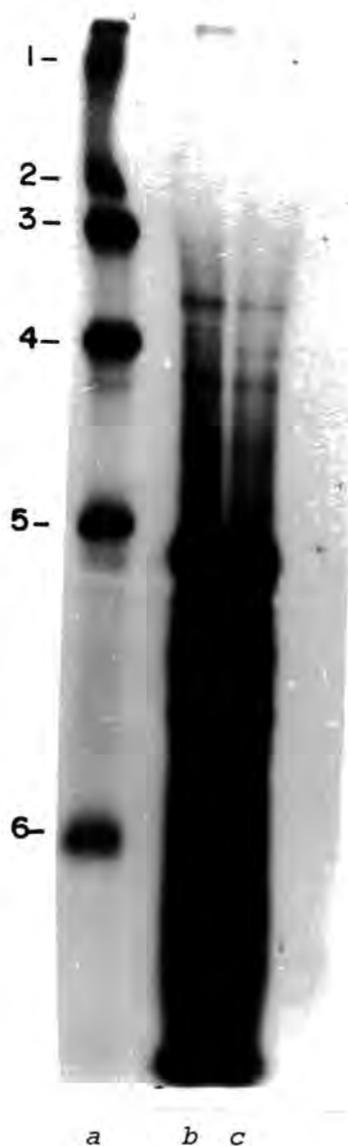


Figure 19. SDS-PAGE of the in vitro translation products in different volumes of the reaction mixture (track b, 10  $\mu$ l and track c, 20  $\mu$ l). The numbers in track a indicate the position of [ $^{14}$ C] methylated proteins of known molecular weight. These are: 1, myosin (200 000); 2, phosphorylase-b (92 500); 3, bovine serum albumin (69 000); 4, ovalbumin (46 000); 5, carbonic anhydrase (30 000); 6, lysozyme (14 300).



a b c d e f g h i j k l m

Figure 20. SDS-PAGE of the in vitro translation products immunoprecipitated with different antisera: track a, non-immune antiserum; track b, Coix  $\alpha$ -amylase inhibitor/endochitinase antiserum; track c, sugar beet endochitinase antiserum. Tracks d to m represent only increasing volumes of the in vitro translation products (from 1.0 to 2.0  $\mu$ l of the reaction mixture, see section 2.21)

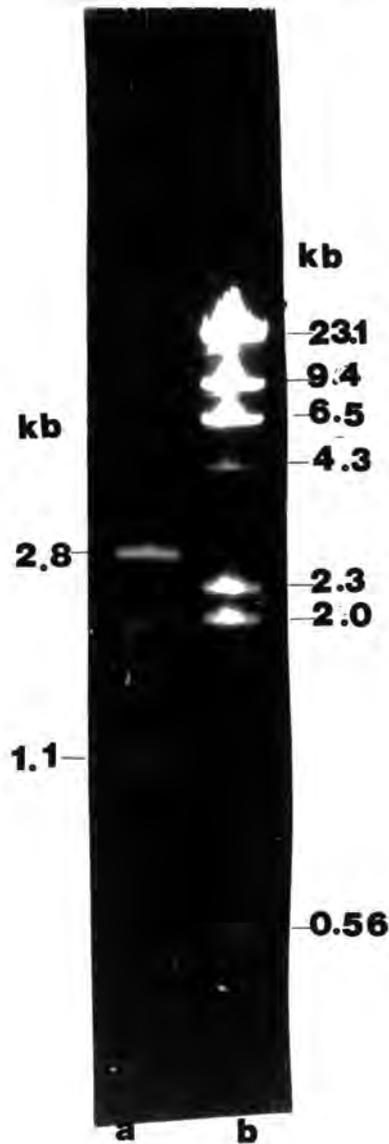


Figure 21. Agarose gel electrophoresis of the bean endochitinase cDNA (pCh18, Broglie *et al.*, 1986) after cleavage with *EcoRI* (track a). The positions of  $\lambda$ -Hind III DNA size markers of known molecular weights are indicated.

**CHAPTER 4**

**GENERAL DISCUSSION AND FUTURE WORK**

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The natural protein inhibitors of enzymes are a heterogeneous group which is widespread in occurrence (Laskowski and Kato, 1980). In plants the chemistry and biochemistry of these proteins, including topics such as their isolation, properties and structure have been studied in great depth (Belitz and Weder, 1990). These inhibitors have been assigned to ten most likely families (Richardson, 1991; see Table 1). Proteinase inhibitors have received particular attention. The majority of proteinase inhibitors known and characterized so far are directed towards serine proteinases (Bode and Huber, 1992). After the well-studied group of serine proteinase inhibitors the next most widely studied group of inhibitors is that which have activity against amylases (Belitz and Weder, 1990). The possible roles of both types of inhibitors as defensive agents against the attacks of insect and microbial pests have also been a great stimulus for much research (Ryan, 1990) and were the main reason for the present study, which was concentrated on the seeds of the relatively little-studied cereal Coix lachryma-jobi.

Protein inhibitors of proteinases have been isolated and characterized from cereal grains such as wheat, barley, ragi, corn, rye, oat and foxtail millet (Boisen, 1983; Lyons et al., 1987; Tashiro et al., 1990; Poerio et al., 1989, Campos and Richardson, 1983). Although these cereal proteinase inhibitors can be seen to belong to several of the families

mentioned above, quite a number of them can be ascribed to the Bowman-Birk family, which was originally believed to contain only proteins from the Leguminosae. The cereal proteinase inhibitors in this family can be divided into two types. One type comprises single-headed inhibitors, such as the wheat trypsin inhibitor II (Odani et al., 1986) with a molecular mass of about 7000, and the other type comprises double-headed inhibitors composed of two tandemly repeated sequences, such as the wheat germ trypsin inhibitor I (Odani et al., 1986).

The first part of the present study describes the purification, characterization and sequence determination of a major trypsin inhibitor (TI1) of the Bowman-Birk type from the seeds of the cereal Coix lachryma-jobi. This protein was first isolated by Ohtsubo et al. (1985). These workers suggested that this protein was of the double-headed tandem type, but were unable to confirm this by any sequencing studies. The present study used additional methods of purification (preparative reverse phase HPLC) to obtain a homogeneous product suitable for amino acid sequencing. The complete amino acid sequence was determined by the manual DABITC-PITC double coupling method applied to peptides derived from the reduced and S-carboxymethylated protein by separate enzymic digestions with trypsin, chymotrypsin and the Glu-C specific endoproteinase from S. aureus V8. The overlapping nature of these peptides was sufficient to establish that the Coix TI1 consisted of 64 amino acid

residues, corresponding to a calculated molecular mass of 7262 Da. Thus it was shown that Coix TI1 is a single-headed inhibitor of the Bowman-Birk type like the wheat germ trypsin inhibitor II (Odani et al., 1986) and is not of the double-headed tandem type as suggested by Ohtsubo et al.(1985).

A highly similar trypsin inhibitor of the Bowman-Birk type was recently demonstrated in seedlings of maize, a species closely related to Coix, by Eckelkamp et al. (1993). These workers showed that the mRNA transcript for this protein was induced in isolated coleoptile segments by the injury caused in cutting segments, but not by auxin. Using cDNA as a hybridisation probe it was shown that wound-induced transcripts accumulated in isolated segments from mesocotyls, primary leaves and roots as a systemic response to a wound signal which was translocated in a polar manner (from the bottom to the top). It would be interesting to discover whether the Coix Bowman-Birk inhibitor described here is also induced in a similar manner by wounding.

The second part of this present work describes the purification, characterization and partial sequencing of a novel inhibitor of locust gut  $\alpha$ -amylase from seeds of Coix lachryma-jobi. The inhibitor consists of two isomers, each of which appears to be a dimer of closely similar or identical subunits of about 26400 molecular mass, and associated by interchain disulphide bonds. Assay of the major isomer with other amylases from five different sources (human saliva,

porcine pancreas, Bacillus subtilis, Aspergillus oryzae and barley malt) showed no inhibitory activity in contrast to the inhibitory specificities of many other cereal  $\alpha$ -amylase inhibitors which may inhibit a range of heterologous amylases (Garcia-Olmedo et al, 1987). It would be very interesting if future work could determine whether this protein does in fact inhibit a wider range of  $\alpha$ -amylases in addition to the enzyme from the gut of locust(Orthoptera). In particular it should be tested against the enzymes from other insects such as the coleoptera Tribolium, Tenebrio, and Callosobruchus, and the lepidoptera, Heliothis, Spodoptera and Manduca which are important agricultural pests, but it would also be pertinent to know if there was any activity against plant  $\alpha$ -amylases, and also whether any endogenous  $\alpha$ -amylase enzymes in the plant tissues of Coix were inhibited.

The partial amino acid sequence of this Coix inhibitor of locust  $\alpha$ -amylase had no similarities (homology) with any other presently known inhibitors of  $\alpha$ -amylases or proteinases and therefore cannot be assigned to any of the existing families of enzyme inhibitor proteins. It would obviously be of interest to discover whether similar proteins with this inhibitory activity towards amylase exist in other plant tissues (see later in this discussion).

Whilst the partial amino acid sequence of the Coix protein had no similarities with other known inhibitors, a search of protein sequence databases using computer-based

methods revealed that it had a high degree of homology with partial and complete amino acid sequences of a number of endochitinase enzymes from sources such as barley, bean, potato and tobacco (Leah et al, 1987; Kragh et al, 1991; Broglie et al, 1986; Gaynor, 1988; Shinski et al, 1987; van Huijsduijen et al, 1987). This surprising finding was substantiated later during the investigation when it was demonstrated that the Coix protein did in fact have enzyme activity as an endochitinase and could release radioactivity when incubated with tritium-labelled chitin. Clearly it is very tempting to suggest that some or all of these other endochitinases which have sequence homology with the inhibitor of locust gut  $\alpha$ -amylase from Coix seeds may also inhibit this enzyme from locusts or other insects. The answer to this question will have to come from future work (see suggestions for further work at the conclusion of this discussion).

Most endochitinases, including the Coix bifunctional protein reported here, have Mr values estimated by SDS-PAGE of between 25000 and 30000. They can be classified into subgroups with low and high pI values, called 'acidic' and 'basic' endochitinases respectively. The enzyme which was purified during this study is clearly of the basic type (pI 8.5-9.0). Other workers have recently reported another chitinase from Coix but this enzyme was an exochitinase and had a molecular mass of only 10kDa (Zhe-fu et al, 1992). It should also be noted that although this study only

provided limited sequence information about the N-terminal region of the Coix endochitinase (see peptides C21, C29, C34 and T32 shown in Fig. 17) what evidence there is indicates that the Coix protein belongs to the class I chitinases as defined by Shinshi et al. (1990). These class I chitinases are basic proteins that contain a highly conserved cysteine- and glycine- rich N-terminal domain with putative chitin binding properties, and they are generally located in the plant cell vacuole (Broekaert et al., 1989; Lucas et al., 1985; Shinshi et al., 1990; Stanford et al., 1989; Wright et al., 1984).

The active site in maize chitinase has been tentatively localized by selective modification of an essential tyrosine residue (Verburg et al., 1992). This residue corresponds to the first tyrosine in the sequence N-Y-N-Y-G which is conserved in most basic endochitinases (see Fig. 17). It is interesting to note that good evidence was obtained during the present investigation for the existence of this sequence in the structure of the Coix protein (see peptides T25, T44 and C19 in Fig. 17).

Thus the Coix amylase inhibitor/ endochitinase protein isolated during this investigation is a further example of an inhibitor with more than one possible function. Other examples of bifunctional inhibitors have been found in seeds of ragi (Campos and Richardson, 1983; 1984), maize (Richardson et al., 1987), barley (Mundy et al., 1983), wheat (Warchalewski, 1987) and rice (Ohtsubo and

Richardson, 1992).

The Coix protein is however different from the above bifunctional inhibitors in that it is the first protein to be discovered with activity both as an inhibitor (of locust  $\alpha$ -amylase) and as a hydrolytic enzyme (endochitinase).

Furthermore there is also a possibility that it is actually trifunctional, as many of the lysozymes which have been purified from plants such as from the latex of papaya, fig and *Hevea brasiliensis* (Howard and Glazer, 1969; Glazer et al, 1969; Tata et al, 1983; Jekel et al, 1991), Rubus cell cultures (Bernasconi et al, 1985), turnip (Bernier et al, 1971) and cucumber (Majeau et al, 1990) have been shown to be active as endochitinases. The intriguing possibility that the Coix protein acts as a lysozyme remains to be tested.

Whatever the outcome is of such further tests, our present knowledge of the Coix  $\alpha$ -amylase inhibitor/ endochitinase strongly suggests it may have considerable potential as a defensive agent against the attacks of both fungal and insect pests, and might therefore be a useful target for the production of transgenic plants via genetic engineering.

Against insects the Coix protein might be active in two defensive ways. Firstly it may inhibit their digestion of plant food (starch) by inhibiting the  $\alpha$ -amylases in their alimentary canals; and secondly it might have a more profound affect on their general metabolism by attacking their chitin. Chitin is present not only in the exoskeleton

of insects but is also an important component of the peritrophic membrane which lines their guts (Chapman, 1969). This peritrophic membrane is usually a cylindrical sheet that envelops the food in the midgut. It consists of a network of chitin in a protein-carbohydrate matrix (Richards and Richards, 1977), and plays important roles in digestion, such as a mechanical protection for the midgut cells, as a physical barrier against micro-organisms, as a device to lower the losses of the digestive enzymes in excretion, and a permeability barrier for digestive enzymes and the products of digestion (Terra, 1990). Clearly any damage caused to this membrane by ingested endochitinase is likely to be detrimental.

Chitin is also a component of fungal cell walls and it is known that chitinases can inhibit the growth of many fungi *in vitro* by causing lysis of hyphal tips, especially in combination with  $\beta$ -1,3-glucanase (Broekaert et al., 1988; Broglie et al., 1991; Mauch et al., 1988; Schlumbaum et al., 1986; Collinge et al., 1993). Furthermore chitinase has been shown to accumulate around fungal hyphal material *in vivo* (Benhamou et al., 1990; Wubben et al., 1992).

Endochitinases have been reported in healthy tissues of a number of other plant species such as beans, wheat, tomato, barley, soybean, oats, potato and yam (Powning and Irzykiewicz, 1965; Leah et al., 1987; Molano et al., 1979; Pegg and Young, 1982; Wadsworth and Zikahis, 1984; Fink et al., 1988; Gaynor, 1988; Araki et al., 1992). They may also be

produced in response to damage or invasion by pathogens (Pan et al., 1992; Nasser et al., 1988; Schroder et al., 1992; van Huijsduijzen et al., 1987; Broglie et al., 1986; Metraux and Boller, 1986; Kombrink et al., 1988), or induced by treatment with growth regulators such as ethylene (Mauch et al., 1992) or gibberellic acid (GA3)(Swegle et al., 1989). These findings have confirmed the inclusion of endochitinases amongst the set of so-called pathogenesis-related (PR) proteins which are thought to be involved in plant defense.

Thus there is good reason for thinking that the Coix protein (via its gene) might be a good target for transfer to other plants in attempts to increase their resistance to insect pests and fungal pathogens. However, before this is done its potential ought to be tested further by in vivo feeding tests with selected insects and in growth/culture experiments with the fungi. For example, the purified protein could be incorporated into artificial diets and fed to locusts (and/or other insects) in a manner similar to that employed by Gatehouse and Boulter (1983) in their examination of the effects of cowpea trypsin inhibitors on the growth, development and survival of larvae of the bruchid beetle (Callosobruchus maculatus). Similarly the effects on fungi could be assayed by using methods similar to those used by Roberts (1986) and Roberts and Selitrennikoff (1988) in investigating the anti-fungal effects of endochitinases from barley, maize and wheat.

In order to carry out any insect feeding trials and

growth tests with fungi, it is probable that larger amounts of the purified Coix protein will be required than were obtained during the present investigation. Also future attempts to complete the sequence determination on this protein will require further material (see later discussion). Whilst the methods used here (affinity chromatography on Red Sepharose and reverse phase HPLC) gave a rapid purification of the required protein in a homogeneous form the yields were relatively low. It is possible that larger quantities might be purified by replacing the Red Sepharose step by a more specific affinity chromatography on columns of immobilized chitin as described by Ride and Barber (1990) for the isolation of the multiple forms of endochitinase from wheat leaves. A further possibility would be to isolate cDNA clones for this protein and express them in E. coli or another heterologous system. (see later in discussion).

One of the objectives of this study was to isolate and characterize the cDNA's and genomic clones for the proteins of interest. However this was not possible in the time available. After the work described in this thesis was concluded another worker at the University of Durham attempted to isolate the cDNA and genomic clones for the Coix  $\alpha$ -amylase inhibitor/ endochitinase (Fairweather, 1993). This worker employed antibodies raised against the protein and mRNA preparations provided by the present investigation. One of the approaches attempted by Fairweather (1993) was

the amplification of the putative  $\alpha$ -amylase inhibitor/ endochitinase cDNA and genomic sequences using the polymerase chain reaction (PCR). The PCR primers used for amplification were designed from selected peptides from the relatively conserved regions of the C and N termini of the partial protein sequence described in this thesis. Despite lengthy optimization procedures to increase the stringency of amplification the  $\alpha$ -amylase inhibitor/ endochitinase gene was not amplified.

One possible explanation for this lack of success was that only part of the protein sequence was available from this investigation. This highlights the importance of further work to determine the complete amino acid sequence of the Coix protein. Future attempts should note the various findings of this study:

i). The N-terminus of the protein appeared to be blocked as the intact protein gave no DABTH-amino acid when subjected to the manual DABITC/PITC sequencing method and no peptide(s) were sequenced which corresponded to the N-terminal regions of other endochitinases (see p. 108). Examination of the homologous sequences shown in Fig. 17 suggests that the most likely reason for the blocked N-terminus might have been the presence of a pyroglutamic acid (pyrrolidone carboxylic acid) formed by the cyclization of an N-terminal glutamine. If this is the case, then treatment of the reduced and S-carboxymethylated protein with the enzyme pyroglutamate aminopeptidase (Aitken et al., 1989) should

remove the blocked amino acid and permit direct automated N-terminal sequencing. If, on the otherhand the N-terminal amino acid was acetylated it could be removed by treatment with the acyl peptide hydrolase (Farries et al., 1991) which has recently become available commercially.

ii). The various peptides produced by enzymatic means (with trypsin and chymotrypsin ) were only obtained in relatively low yields which prevented extended numbers of cycles of sequencing. This difficulty might be overcome by using larger quantities of starting material than the 10mg amounts used in this work.

iii). Many of the tryptic and chymotryptic peptides were only obtained as components of complex mixtures, and could not therefore be sequenced without ambiguity (eg. the gaps in the sequence shown in Fig. 17). This was due to the fact that the Coix  $\alpha$ -amylase inhibitor/ endochitinase is a relatively large protein (approximately 240 amino acids) and gave rise to relatively large numbers of peptides when digested by either trypsin or chymotrypsin. Although it was possible to purify all of the tryptic and chymotryptic peptides from the smaller (64 amino acid) Coix trypsin inhibitor by analytical reversed phase HPLC this technique was unable to resolve all of the components in the complex mixtures from the larger protein. Future work should employ other ancillary techniques of peptide purification such as preliminary fractionation by gel filtration on a molecular sieve like BioGel P-6 (Richardson et al., 1984), and if

required ion-exchange HPLC techniques (Cordeiro et al., 1993) after the reverse phase HPLC.

iv). The sequence similarity of the Coix protein to other endochitinases shown in Fig. 17 suggests that the protein might contain a small number of methionine and tryptophan residues located in convenient positions in the overall sequence. Chemical cleavage with cyanogen bromide (Aitken et al., 1989) and o-iodosobenzoic acid (Aitken et al., 1989) respectively should generate useful large fragments which could be sequenced by automatic means.

v). If the above improved methods fail to reveal the complete sequence then other enzymes such as the Staphylococcus aureus V8 endoproteinase (specific for Glu-C and Asp-C bonds) or pepsin (specific for the peptide bonds involving large hydrophobic amino acids such as Leu, Phe and Tyr )might be used to obtain essential information.

Bowles (1990)( see section 1.2) has reviewed the three different classes of proteins, which together with other chemicals (see p.3) are believed to constitute a complex array of defenses used by plants against the attacks of pests and pathogens. The enzyme inhibitors and endochitinases such as the Coix protein described here are included amongst the class II proteins.

Transgenic plants provide an opportunity to test whether such proteins can play a role in defense. The main objective of plant genetic engineering is the introduction of agronomically desirable phenotypic traits (e.g. defensive

proteins) into crop plants in situations where conventional breeding methods have been unsuccessful or are too difficult. Early successes in transformation were confined mostly to dicotyledonous plants such as tobacco, petunia, potato and tomato with relatively few monocotyledonous plants being successfully transformed. However the development of new techniques for gene delivery, such as transformation of protoplasts with DNA using electroporation (Shillito and Saul, 1988) and microprojectile bombardment (Klein et al., 1988) has increased the efficiency of transformation of monocotyledons, particularly of cereal crops. Additionally, the development of efficient methods for the regeneration of plants from tissue cultures via somatic embryogenesis has also contributed to improving the efficiency of transformation of monocotyledonous plants (Kantha, 1992).

The genes coding for protein inhibitors of various enzymes have figured prominently amongst the first attempts to produce transgenic plants. A direct test of the role of the cowpea trypsin inhibitor protein (CPTI) in defending plants against insects was first demonstrated by Hilder et al.(1987). The gene encoding CPTI was utilized to transform tobacco plants which then expressed this protein in their leaves. Transformed plants were more resistant to feeding by the herbivorous larvae of Heliothis virescens (order, Lepidoptera) than untransformed control plants. Further trials in California showed that the expression of CPTI in

tobacco gave significant protection in the field against Helicoverpa zea (order, Lepidoptera) (Hoffman et al., 1992), but this was not as efficient as when the plants were transformed with the  $\delta$ -endotoxin gene from Bacillus thuringiensis.

Other workers (Johnson et al., 1989) transferred genes encoding tomato inhibitor I (having only chymotrypsin inhibitory activity) and the genes encoding tomato and potato inhibitor II proteins (having both chymotrypsin and trypsin inhibitory activities) to tobacco plants. The growth of larvae of Manduca sexta (tobacco hornworms; order, Lepidoptera)) feeding on leaves of transgenic plants expressing Inhibitor II was significantly retarded compared to the growth of the larvae fed only on untransformed leaves. The presence of tomato Inhibitor I in transgenic tobacco leaves had little effect on the growth of the larvae. These experiments indicated that trypsin inhibitory activity was more important than chymotrypsin inhibitory activity in the inhibition of larval growth. On the other hand very recent results obtained by McManus et al. (1993) showed that transgenic tobacco plants expressing potato proteinase inhibitor II, were most active against chymotrypsin-like proteinases, had adverse effects on growth of larvae of the noctuid lepidopteran Chrysodeixis eriosoma (green looper), but were not as effective against other insects such as Spodoptera litura (Lepidoptera). In other words these results illustrate the fact that the type of

digestive protease activity present in a target insect species has to be known and used in the design of transgenic plants.

This important consideration has been stressed in a recent review by Gatehouse et al. (1993) on the various possible approaches to insect resistance using transgenic plants. There have been several suggestions that the thiol-proteinases of the cystatin type are the important digestive proteinases in some insect species (Gatehouse et al., 1985; Liang et al., 1991). A protease inhibitor of this type from rice, oryzacystatin, has been expressed in poplar trees to give resistance to the coleopteran insect pests Chrysomela populi and Chrysomela tremula, and in oilseed rape with the intention of conferring resistance to the seed weevil Ceuthorrhynchus (Coleoptera) (Jouanin et al., 1993, unpublished results reported by Gatehouse et al., 1993). On the other hand northern blot analyses of the levels of expression of inhibitors of this type in resistant and susceptible lines of cowpea seeds by Fernandes et al. (1993) indicated that the resistance to bruchid beetles was not related to the levels of cysteine proteinase inhibitors.

So far there has been relatively little investigation of the possible use of genes coding for  $\alpha$ -amylase inhibitors in transgenic plants. There is strong circumstantial evidence that an  $\alpha$ -amylase inhibitor ( $\alpha$ -AI) from bean (Phaseolus vulgaris) seeds is encoded by a previously identified lectin gene whose product is referred

to as a lectin-like protein (LLP). Altabella and Chrispeels (1990) have constructed a chimeric gene consisting of the coding sequence for LLP with the 5' and 3' flanking sequences of the lectin gene that encodes phytohaemagglutinin-L (PHA-L). This chimeric gene was expressed in transgenic tobacco seeds and an extract of the seeds of transformed tobacco plants inhibited pig pancreatic  $\alpha$ -amylase activity as well as the  $\alpha$ -amylase present in the midgut of the insect pest Tenebrio molitor. In considering the possible exploitation of the genes for  $\alpha$ -amylase inhibitors as targets for genetic engineering into transgenic plants it is relevant to note that they may have more protective effects against Coleoptera than against the Lepidoptera. This is because the pH optimum for the formation of the complex between the amylase enzyme and the inhibitor is in the range of 5-6. The mid-guts of Coleoptera have an acidic pH whilst the Lepidoptera are usually basic (Altabella and Chrispeels, 1990).

Most of the initial work on the possible use of chitinase genes in transgenic plants has concentrated on bacterial chitinases as the source of the transferable gene (Sundheim et al., 1988; Jones et al., 1986, 1988; Lund et al., 1989; Jach et al., 1992). However Roberts and Selitrennikoff (1988) have shown that the microbial enzymes which are exochitinases are relatively ineffective as antifungal agents and have suggested that endochitinases from plants are likely to be more successful in transgenic plants.

Transgenic tobacco and rape plants containing a bean chitinase gene with a constitutive promoter have been shown to exhibit higher basal levels of chitinase and concomitant increased resistance to Rhizoctonia solani when compared to control plants (Broglie et al., 1991). On the other hand, Neuhaus et al.(1991) have transferred a tobacco endochitinase gene to Nicotiana sylvestris . Despite being correctly processed to produce enzymatically active chitinase levels which were up to 120 fold higher than in non-transformed plants, the transformed plants did not appear to have substantially increase resistance to the chitin containing fungus Cercospora nicotiana which causes frog eye disease. In interpreting this negative result it should be remembered that usually endochitinases and  $\beta$ -1,3- glucanases are coordinately induced by ethylene or by the attacks of pathogens on plants (Kirsch et al., 1993). This has led some workers (Mauch et al., 1988a,1988b; Schlumbaum, 1986) to suggest that endochitinase may only be an effective fungicide in vivo when in the presence of  $\beta$ -1,3-glucanase. Clear evidence that the class I endochitinase and  $\beta$ -1,3-glucanase enzymes in tobacco acted synergistically against the fungus Fusarium solani both in vivo and in vitro has been provided recently by Sela-Burlage et al.(1993).

In general, plant hydrolases (e.g. chitinases and  $\beta$ -1,3-hydrolases) with potential functions in disease resistance are located in the vacuole or the cell wall space (Matile, 1984). Furthermore, specific knowledge of

vacuolar targeting information for plant proteins is now being gained. A number of studies have demonstrated that plant proteins have a vacuolar sorting signal included as part of their primary amino acid sequence (Chrispeels and Raikhel, 1992; Melchers et al., 1993) . In the case of the tobacco chitinases the vacuolar form has the C-terminal sequence Gly-Leu-Leu-Val-Asp-Thr-Met, and this domain is absent from the homologous cell wall or extracellularly targeted chitinases (Shinshi et al., 1990; Melchers et al., 1993; Sela-Burlage et al., 1993). Similarly, a C-terminal extension of 12 amino acids, of which the last 7 are the heptapeptide above, is sufficient to redirect cucumber chitinase, which is normally secreted, to the vacuole (Neuhaus et al., 1991).

Unfortunately the partial sequence of the Coix amylase inhibitor/ endochitinase determined during the present work does not extend to the C-terminus (Fig. 17). When details of the complete sequence are available (see earlier discussion) the presence or absence of this C-terminal extension in the Coix protein will hopefully indicate its likely targeting. Alternatively, immunocytochemical methods could be used to determine the localization of the protein (Van den Bosch, 1986). The antibody raised against the Coix protein during this work could be used as an affinity probe for the antigen exposed at the cut surfaces of sections. Colloidal gold particles, conjugated to secondary antibodies or to protein A, could then be attached to the bound primary antibody and

thus serve as electron-opaque markers of the antigen when the section is examined in the electron microscope. Some idea of the tissue-wide distribution of the Coix protein might also be obtained from light-microscopic visualization of the antibody-gold labelling of the antigen in sections of other tissues (leaves, roots, etc). However in most cases the antigen of interest would not be sufficiently concentrated for the gold particles to be seen in the light microscope without further amplification such as silver enhancement (Van den Bosch, 1986).

Various other methods have been used to study the location and differential expression of gene products in different tissues during development. For example, the widely used technique of western blotting involves the immunodetection of specific proteins adsorbed to blotting media such as nitrocellulose and Immobilon membranes (Ono and Tuan, 1990). Similarly information on mRNA populations and gene expression can be obtained from DNA-RNA hybridization (northern blotting) experiments, and details of the families of encoding genes from southern blotting (DNA-DNA hybridization).

The use of such methods for studying enzyme inhibitors in plants is well illustrated by the work of Fernandes et al. (1993). Total RNA was isolated from developing cotyledons of cowpea seeds that were resistant or susceptible to the attacks of the cowpea bruchid beetle. The cDNA for a cysteine proteinase inhibitor was isolated from a

cDNA library prepared from developing seeds of an insect-resistant line and used as a probe in northern blotting hybridization experiments in order to study changes in mRNA populations during seed development. In both resistant and susceptible lines expression in the cotyledons was highest at 8-10 days after pollination and then declined, although transcripts were still present 18 days after pollination. Southern blotting experiments indicated that small gene families for this protein were present in both resistant and susceptible lines of cowpea. Similarly, as noted above, northern blotting was also used by Eckelkamp et al. (1993) to follow the wound-induced local accumulation patterns of transcripts for the maize trypsin inhibitor which is homologous with the Coix Bowman-Birk trypsin inhibitor.

Other workers (Leah et al., 1991) have used northern blotting to follow the expression of three different seed proteins with anti-fungal properties in barley. Chitinase mRNA accumulated to high levels in aleurone cells during late seed development and early seed germination, while high levels of mRNA encoding a ribosome-inactivating protein (RIP) accumulated only in the starchy endosperm during late seed development, and a glucanase mRNA accumulated in low levels during seed development and to higher levels in aleurone and seedling tissues during germination. Southern blots indicated that the three proteins were each encoded by small families of three to eight genes.

In conclusion, a programme of future research on the

molecular biology of the Coix  $\alpha$ -amylase inhibitor/  
endochitinase might involve some or all of the following:

1. Utilization of the antisera raised against the Coix amylase inhibitor/ endochitinase (or against the sugar beet or wheat germ endochitinases as these antisera were shown during the present work to react strongly with the Coix protein, see section 3.10 and Fig. 18) for:

a). Immunolocalization experiments to detect the Coix protein in tissues other than seeds (e.g. leaves, stems and roots),

b). Immunolocation of related proteins in other plant species,

c). Electron microscopy with immunogold labelling to determine whether the Coix protein is deposited only in vacuoles or is extracellularly excreted,

d). Western blotting to determine the time course of synthesis (and/or degradation) of the Coix protein during seed development and germination.

e). Also to look for possible induction of expression of this protein by wounding, chemicals (e.g. fungal elicitors, growth regulators, salicylic acid etc) and/or infection.

2. Construction of a cDNA library in a plasmid of the pUC type or in phage  $\lambda$  using reverse transcriptase and the poly (A<sup>+</sup>)-rich mRNA isolated from seeds of Coix during this work. This cDNA library could be screened using either the oligonucleotide probes mentioned above or the bean

endochitinase cDNA. The cDNAs would provide complete sequences of the proteins. They would also provide probes for southern blotting (to determine gene copy number), northern blotting (to determine expression patterns) and to isolate corresponding genes.

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