Isolation and Partial Characterization

of

Serine Protease Inhibitors from *Triticum aestivum* cv. Witwol

by

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Summary

The aim of this study was to isolate and characterize serine protease inhibitors from *Triticum aestivum* cv. Witwol, and determine the potential of the isolated protease inhibitors as antifungal and defensive compounds against herbivorous insects. Two subtilisin inhibitors and one trypsin inhibitor were isolated from *Triticum aestivum* cv. Witwol with molecular masses of 13 282, 12 081 and 7 807.1 Da, respectively. The subtilisin inhibitor with molecular mass of 13 282 Da was shown to be homologous to a wheat α-amylase/trypsin inhibitor (Accession number P16159) while the trypsin inhibitor with molecular mass of 7 807 Da was shown to be homologous to a barley subtilisin/chymotrypsin inhibitor (Accession number P16062) as well as a wheat trypsin inhibitor precursor (Accession number Q43667). The trypsin inhibitor was shown to be a competitive inhibitor, and to inhibit bovine trypsin with a K_i value of $13x10^{-6}$M (when using BAPNA as substrate) and a K_i value of $1x10^{-7}$M (when using TAME as substrate). No inhibitory activity could be demonstrated against chymotrypsin when using ATEE as substrate. When tested against *Helicoverpa armigera* gut extract, the isolated trypsin inhibitor inhibited the serine protease activity in the gut extract, with levels which compared favourably with levels of inhibition displayed by Soybean Kunitz Inhibitor. The trypsin inhibitor failed to show any inhibitory activity against any of the fungal isolates tested. The wheat α-amylase/trypsin (P16159) was cloned into the pCR®T7/NT expression vector and a protein with molecular mass of approximately 14 000 Da was expressed after induction with IPTG. The proteins isolated during this study could not be conclusively sequenced and hence final classification was not possible.

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I declare that "Isolation and Partial Characterization of Serine Protease Inhibitors from *Triticum aestivum* cv. Witwol" is my own work and that all sources I have used or quoted have been indicated and acknowledged by means of complete references.

List of Abbreviations

ATEE	N-acetyl-L-tyrosine ethyl ester
BAPNA-HCl	Nα-benzoyl-DL-arginine p-nitroanilide
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
CLA	carnation leaf agar
CNBr	cyanogen bromide
CpTI	cowpea trypsin inhibitor
DMSO	dimethyl sulfoxide
EST	expressed sequence tag
EtBr	ethidium bromide
HPLC	high performance liquid chromatography
JA	jasmonic acid
LTP	lipid transfer protein
MALDI-TOF	matrix-assisted laser desorption ionisation time of flight
Me-JA	methyl jasmonate

PA	phytoalexin
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
PI	protease inhibitor
PGIP	polygalacturonase inhibitor protein
PR	pathogenesis related
RIP	ribosome inactivating protein
SA	salicylic acid
SAR	systemic acquired resistance
SDS-PAGE	sodium dodecyl sulphate
SKTI	soybean kunitz trypsin inhibitor
TAME	Nα-p-tosyl-L-arginine methyl ester
TFA	trifluoroacetic acid
TI	trypsin inhibitor
TMV	tobacco mosaic virus
WGA	wheat germ agglutinin

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Literature Review

Chapter 1
Literature Review

Introduction

Plant diseases and plant pests are major constraints to plant growth and development, resulting in severe crop losses annually. Plant pathogens can be a variety of things, living and non-living. Non-living pathogens exist as physical conditions that plants are exposed to- climatic conditions can cause damage to plants and there are various agricultural practices that can also be harmful to the plant. Living pathogens are called parasitic or infectious diseases and are extremely contagious and can spread from plant to plant very quickly. Insects, nematodes, mites and higher animals can be considered as pathogens, as are slime mold, bacteria, fungi and parasitic higher plants. Viruses and viroids are also considered pathogens (URL 1). Fungal diseases, in particular, severely limit the production of major crops, as do insect crop pests. Effective control of pathogens has led to widespread use of chemical fungicides and insecticides, but with potentially deleterious environmental and human health consequences associated with this practice, strategies to utilize natural host plant resistance mechanisms for disease control are being pursued (Yun *et al.*, 1997). The agrochemical industry has been actively looking for less damaging ways to control insect pests, and has introduced a number of more environmentally friendly pesticides. In addition, alternative strategies for pathogen and pest control have been pursued, such as biological control, and the use of plant varieties with inherent resistance.

1.1 Fungal Pathogens and Proteases

Most plant diseases are caused by pathogenic fungi. Perhaps the most important economic fungal diseases of plants are caused by the rusts and the smuts. Many plant pathogenic fungi exhibit a complicated life cycle and the survival and infectivity of most plant pathogenic fungi depend on prevailing conditions of

temperature and moisture. Economically important fungal pathogens include *Fusarium, Monilinia, Verticillium, Aspergillus and Botrytis.* These organisms cause wilt, seedling blight, brown rot of stone fruit, wilting, storage rot and blossom blight on various plants. *Fusarium culmorum, Gaeumannomyces graminis* var. *tritici, G. graminis* var.*graminis* and *G. graminis* var. *avenae* are pathogens of cereals and grasses. *Gaeumannomyces graminis* var. *tritici* causes take-all in wheat, barley and asymptomatically colonizes the surface of oat roots. *G. graminis* var.*graminis* is a pathogen of rice, wheat and turfgrass. *G. graminis* var. *avenae* is the main cause of oat take-all and is pathogenic for wheat, barley and turfgrass. In temperate regions, *F.culmorum* is a severe pathogen of cereals and grasses, causing root rot and head blight (Friebe *et al.*, 1998).

1.1.1 Fungal Proteases

Extracellular subtilisin-like proteases have been reported from a wide range of fungal species, including saprophytes and pathogens as well as mutualists, and are considered to be important components of pathogenicity. A fungal protease-At1-which is expressed by the fungus *Acremonium typhinum* in endophyte infected plants, is homologous to the subtilisin-like serine proteases produced by fungal pathogens of insects, nematodes and other fungi. The two enzymes most similar to proteinase At1 are from fungal pathogens of nematode eggs and insects. Application of purified protease from the nematode egg pathogen to immature eggs of *Meloidogyne hapla* resulted in an increase of nonviable eggs. Antibodies to protease Pr1 from the entomopathogen *M. anisopliae* inhibited host colonization. The function of protease Pr1 in pathogenesis, therefore, may be to rapidly degrade insect cuticular proteins, thus allowing penetration of the fungus into the host (St. Leger *et al.*, 1997). RNA gel blot and immunoblot analysis revealed that proteinase At1-like enzymes are expressed in other *Acremonium* and *Epichloe* endophyte/grass combinations and fungal expression of such proteases appears to be a general feature of endophyte infection. Protease–deficient mutants of *Beauveria bassiana* (an entomopathogen) had reduced virulence against the migratory

grasshopper, *Melanoplus sanguinipes*. The mycoparasite *Trichoderma harzianum*, produces a protease (Prb1) which is specifically expressed during the mycoparasitic process and is induced by fungal cell walls while the nematode-trapping fungus *Arthrobotrys oligospora* produces an alkaline serine protease, PII, which is capable of immobilizing free nematodes. The available evidence suggests that these proteases are a component of overall pathogenesis, since in none of these systems has the expression of protease been shown (by targeted gene expression) to be absolutely required for pathogenecity. Expression of subtilisin-like proteases is not limited to pathogenic fungi and may in fact be a universal feature of Ascomycete and Deuteromycete fungi. *Tritirachium album* produces the enzyme proteinase K, which is highly homologous to proteinase At1. *T. album* is a saprophytic fungus and proteinase K is believed to serve a nutritional function by degrading proteins. Similar proteases produced by *Aspergillus* species are responsible for the postharvest decay of stored grains (Reddy *et al.*, 1996).

1.2 Bacterial Pathogens and Proteases

Plant pathogenic bacterial species occur in the genera *Mycoplasma*, *Spiroplasma*, *Corynebacterium*, *Agrobacterium*, *Pseudomonas*, *Xanthomonas*, *Streptomyces* and *Erwinia*. These bacteria are widely distributed and cause a number of plant diseases including hypertrophy, wilts, rots, blights and galls (Atlas and Bartha, 1987). Work from several laboratories has suggested that extracellular proteases of phytopathogenic bacteria have a role in disease development. *Xanthomonas campestris* pv *campestris* Pammel is the causal agent of black rot of crucifers and produces two major extracellular proteases, PRT1 and PRT2 (in liquid medium). These two proteases belong to different classes of serine and metallo proteases respectively, and show different patterns of degradation of β-casein, suggesting different peptide bond specificities. In the natural infection process, *X. campestris* pv *campestris* enters the vascular system of the plant through hydrathodes at the leaf margins and is normally restricted to xylem vessels. In contrast, *X. campestris* pv *armoraciae* (McCulloch) Dye and *X. campestris* pv *raphani* are mesophyllic pathogens of

crucifers which cause leaf-spotting diseases. *X. campestris* pv *armoraciae* is unable to invade the vascular system of the plant and causes a necrotic response in the veinlets surrounding the hydrathodes or in the vascular system after wound inoculation. These pathogens also produce an extracellular protease (PRT3) which is different to PRT1 and PRT2 (Dow *et al.*, 1993).

1.3 Insect Pests

Insect pests can cause considerable crop losses. The post harvest damage to seeds of starchy grain legumes by larvae of bruchidds (Coleoptera: Bruchidae) can be quite extensive. Crops of the common bean, the scarlet runner bean, and the tepary bean can be severely damaged by the bean weevil (*Acanthoscelides obtectus*) and the Mexican bean weevil (*Zabrotes subfasciatus*) (Ishimoto and Chrispeels, 1996). Insect predation on chickpea also results in severe losses in chickpea production annually. Most of the losses are caused by the pod borer (*Helicoverpa armigera* Hubner), a polyphagous pest of the developing seeds of several legume species. *H. armigera* feeding on chickpea begins at flowering and is greatest between 25 and 45 days after flowering. Typically, a single larva damages over five pods per day, leading to heavy losses in crop yield (Giri *et al.*, 1998). Fall armyworm (*Spodoptera frugiperda* [J. E. Smith]) and southwestern corn borer (*Diatraea grandiosella* Dyar) are serious insect pests of corn (*Zea mays* L.) in the southern United States. Larvae of both species damage plants by feeding on leaves within whorls. Other Lepidopteran pests include the sugarcane borer (*Diatraea saccharalis* [Fab]), the corn earworm (*Helicoverpa Zea* [Boddie]) and the European corn borer (*Ostrinia nubilalis* [Huber]) (Jiang *et al.*, 1995). In Australia, *H. punctigera* and *H. armigera* are major pests of several important crops including cotton (URL 3). Many mites, for example spider mites, found on agricultural crops are also major economic pests (URL 4).

1.4 Nematodes

Plant parasitic nematodes are a diverse group of microscopic organisms that feed on the living cells of their host plants. Most species are root parasites and all agricultural systems are affected by nematode problems, with all of the world's most important crops being damaged by one or more species (Burrows *et al.*, 1998).

1.5 Viruses

Many plant pathogenic viruses are classified according to their ability to cause a particular disease. Vectors are important in the transport of viruses that occur in the soil or in diseased plant tissues of susceptible host plants. Various insects, nematodes and fungi can act as vectors for viral diseases of plants (Atlas and Bartha, 1987).

1.6 Plant-pathogen Interactions

1.6.1 Elicitors

Plants can be immunized against disease- causing pathogens or feeding pests. This phenomenon is the result of the development of systemic acquired resistance (SAR). SAR to pathogens usually develops after the appearance of a necrotic lesion around the inoculation site. This localised cell suicide is called the Hypersensitive Response (HR). While the HR effectively traps pathogens in and around lesions, it makes the whole plant more resistant to a wide range of disease-causing microorganisms. Although not as extensively documented as SAR to pathogens, SAR to pests was shown in some cases to be an effective deterrent to continued herbivory (Enyedi *et al.*, 1992). Recognition plays a central role in the interaction between plants and their pathogens. For successful penetration and infection, pathogens must be able to recognise host plants present in the environment and must also be able to recognise and overcome host defense responses. Plants should be able to detect pathogens in their environment and be able to activate defense mechanisms. Elicitors are molecules released or generated during microbial entry and include

oligosaccharide fragments of fungal (Hepta-β-glucoside; oligochitin and oligochitosan) and plant (oligogalacturonide) cell wall polysaccharides (Hahn, 1996). Examples of plant defense mechanisms induced by elicitors include synthesis and accumulation of phytoalexins, the induction of cell death, the production of glycosylhydrolases, the synthesis of proteins that inhibit degradative enzymes produced by pathogens, the production of activated oxygen species and the modification of plant cell walls (Hahn, 1996). Plants rely on transmissible signal molecules that, at low concentrations, can achieve resistance mechanisms in cells not directly invaded by the pathogen or damaged by the pest. Signal molecules in SAR should be synthesized by the plant; increase systemically following attack by a pathogen or pest; move throughout the plant; induce defense-related proteins and phytochemicals; and enhance resistance to pathogens or pests. Endogenous molecular signals in SAR include salicylic acid (SA), systemin, jasmonic acid (JA); methyl jasmonate (Me-JA) and ethylene (Enyedi *et al.*, 1992).

1.6.1.1 Salicylic acid (SA)

SA is widely distributed in monocot and dicot plants, and plants are most likely to synthesize SA from cinnamic acid (the product of phenylalanine ammonia lyase (PAL) – a key regulator of the phenylpropanoid pathway) activity. Exogenous application of SA stimulates resistance to a wide variety of lesion-inducing viral, bacterial, or fungal pathogens. SA induced protection is accompanied by the transcriptional activation of pathogenesis related (PR) protein genes. SA also induces peroxides, superoxide dismutase, and glycine-rich wall proteins, all of which are commonly induced by pathogens and are thought to function in defense (Enyedi *et al.*, 1992).

1.6.1.2 Jasmonic Acid and Methyl Jasmonate

JA and its methyl ester, Me-JA are naturally occurring compounds found in many plant species. JA and Me-JA are implicated as wounding-activated endogenous signals that may alert undamaged tissues of

neighbouring plants of an impending pest attack. Treatment of plant cells with Me-JA leads to an increase in the expression of genes associated with the synthesis of compounds related to defense. Exposure of tomato leaves to gaseous Me-JA elicits plant defense responses similar to those induced by insect feeding or wounding. At a dose of 10nL, gaseous Me-JA induced the synthesis of protease inhibitors I and II in the leaves of healthy tomato plants. Me-JA can also induce the expression of the systemin gene. Thus, the effects of Me-JA and JA on the transcriptional induction of protease inhibitors I and II may be mediated by systemin (Enyedi *et al.*, 1992).

1.6.1.3 Ethylene

Ethylene is a gaseous plant hormone that regulates many physiological processes in plants. Interest in ethylene involvement in SAR came from the observation that inoculation with pathogens increased ethylene evolution from the host plant. Ethylene induces several PR proteins believed to play a role in plant defense. A basic chitinase and β-1, 3- glucanase were induced by ethylene in various plants. Transgenic tobacco and canola plants constitutively expressing an ethylene-inducible bean chitinase, showed enhanced resistance to the chitinous soilborne fungus *Rhizoctonia solani*. Ethylene may also be involved in the strengthening of the cell wall that follows a pathogen or pest attack. Ethylene induces lignification of swede root, possibly by activation of PAL and peroxidase. In addition, cell wall cross-linking hydroxyproline-rich glycoproteins accumulate in the tissues treated with ethylene. However, all structural changes induced by ethylene are usually restricted to the immediate vicinity of the infection or wounding site and therefore are not likely to contribute to SAR (Enyedi *et al.*, 1992).

1.6.1.4 Peptides

Peptides are probably the most commonly used signal molecules in animal systems. Signalling peptides have also been identified in plants, examples of these being CLAVATA 3, phytosulfokines, Enod 40 and systemin (Bisseling, 1999).

1.6.1.4.1 Systemin

An 18-amino acid polypeptide, called systemin, has been identified that activates over 15 defense genes in leaves of tomato plants when supplied at low levels. Systemin (released upon wounding by herbivore attack) is the only peptide shown to possess a hormone-like regulatory function in plants. It is derived from a larger precursor protein, called prosystemin, by limited proteolysis. Prosystemin was found to be as biologically active as systemin when assayed for protease inhibitor induction. Systemin has been shown to be phloem mobile and to be an essential component of the wound-inducible systemic signal transduction system leading to the transcriptional activation of defense genes. A systemin-binding protein in plant plasma membranes has been identified as a receptor of systemin. The signal transduction pathway that mediates systemin signalling involves linolenic acid released from membranes and subsequent conversion to jasmonic acid, a potent activator of defense gene transcription (Schaller *et al.*, 1995).

1.6.1.4.2 Bestatin

Schaller *et al.* (1995) reported that bestatin (an inhibitor of some aminopeptidases in plant and animals) when supplied to tomato plants through their cut stems, is a powerful inducer of the same group of defense genes that are activated in response to wounding and systemin, and may be exerting its effects at or near the

level of transcription of these genes. Bestatin induced the accumulation of PIs I and II in leaves to levels equal to those induced by systemin.

1.6.2 Mechanism of Penetration

Potential fungal invaders encounter the cuticle (outer layer) first and usually penetrate by mechanical force. Penetration of the host plant by an invading fungus gives rise to the potential establishment of physiological contact between the two organisms. Physical penetration can occur through natural openings, wounds or direct penetration. Chemical penetration mechanisms include biochemical attack on plant tissues and cells (Mendgen *et al.*, 1996).

1.6.2.1 Physical Penetration

This can occur through natural openings (unprotected and vulnerable to invasion by fine fungal hyphae) eg. stomata, hydrathodes and lenticels; wounds; direct penetration (which requires a combination of mechanical force and enzymatic pre-softening of the cuticle) and chemical penetration. Once in close proximity to the plant cell wall, many pathogenic fungi are able to launch biochemical attacks on plant tissues and cells to aid and extend penetration (Mendgen *et al.*, 1996).

1.6.2.2 Morphogenesis of Penetration

Fungi produce infection structures that enable the organism to penetrate different types of plant cell walls. The morphogenetic events leading to formation of the infection structures often depend on specific signals provided by the plant surface and are prerequisites for a particular mode of penetration. Physiological changes such as targeted secretion of enzymes or an increase of pressure within the infection structure support the penetration process (Mendgen *et al.*, 1996). In this regard, pathogenic fungi exhibit various degrees of specialization.

Hyphae of *Cladosporium fulvum* increase in diameter immediately after growth through open stomata of hosts; root pathogens such as *Fusarium oxysporum* or *Rhizoctonia solani* accumulate hyphae that may form infection cushions before individual hyphae penetrate, with minor modifications to their morphology. Leaf pathogens such as *Colletotrichum* spp or *Magnaporthe grisea* produce germ tubes that differentiate melanized appressoria from which penetration hyphae develop.

Directly penetrating fungi that do not differentiate appressoria need cell wall degrading enzymes for penetration. In *Fusarium solani* f. sp. *pisi*, dihydroxy-C16 and trihydroxy C-18 fatty acids (which are released from the cutin polymer by low levels of constitutively expressed cutinase), together with soluble nuclear protein factors, were shown to activate cutinase gene transcription. In culture media containing purified plant cell walls or polymers such as pectin, polygalacturonic acid, cellulose, xylans and others, many fungi synthesize an array of enzymes required to degrade these carbon sources. The presence of a single polymer such as polygalacturonic acid is often sufficient to induce a number of different enzymes eg. polygalacturonases, pectin and pectate (polygalacturonate) lyases, and pectin methylesterases.

In the broad host range pathogen *Botrytis cinerea*, pectin not only induces pectic enzyme, but also serves as a second inducer of laccase synthesis and secretion. Laccases are thought to contribute to degradation of lignin, a polymer incorporated into plant cell walls in response to pathogen attack. So penetration in the area of the appressorial pore (a pore in the middle of the appressorial base) is likely to be supported by enzymes that soften the host cell wall. In *Uromyces viciae-fabae* differentiation of appressorium and penetration hyphae is accompanied by the secretion of a complex pattern of lytic enzymes including chitin deacetylase, proteases, acidic cellulases, pectin methylesterases, neutral cellulase, polygalacturonate lyase and amino acid permease, with chitin deacetylase, proteases and acidic cellulases being produced during stomatal penetration. During appressorium formation, several extracellular proteases appear. These proteins show specificity for fibrous hydroxyproline-rich proteins, reminiscent of structural plant proteins that are incorporated into the cell wall in response to fungal attack (Mendgen *et al.*, 1996).

1.6.2.3 Degradation of Plant Cell Wall Components

Pectic polymers are important plant cell wall structural components and many fungi liberate pectic enzymes which have an important role in cell wall degradation. Pectinase or pectolytic enzymes solubilise pectin chains. Hydrolytic polygalacturonases break the linkages between galacturon molecules, and pectin lyases remove water from the linkage to split the chains which result in cell wall maceration. These enzymes are key factors in soft rot diseases such as apple rot diseases caused by *Penicillium expansum* and *Sclerotinia fructigena*. The break down of hemicelluloses requires a mixture of enzymes, known as hemicellulases. Cellulose is degraded by a series of enzymatic reactions with cellulose degrading enzymes being important in wilt diseases caused by *Fusarium oxysporum* and *Verticillium albo-atrum* (Mendgen *et al.*, 1996).

1.7 Plant Defense Mechanisms

Plants are continually exposed to insects, nematodes and other potentially damaging pests, as well as to a wide variety of parasitic microorganisms. Yet, the majority of plants remain healthy most of the time. This observation suggests that plants must possess highly effective mechanisms for preventing parasitism and predation, or at least limiting their effects. Plants have evolved a variety of different mechanisms to cope with this constant threat by phytopathogenic microorganisms. They have physical barriers and antimicrobial compounds that are preformed in advance of pathogen attack, and after infection by pathogens, these constitutive defenses are supported by induced mechanisms. Cell walls can be reinforced by for example oxidative cross-linking, and hypersensitive cell death is triggered to isolate the pathogens from the uninfected parts of the plant. The induced proteins include enzymes involved in lignification and in the synthesis of phytoalexins, PR proteins, hydrolases (chitinases and 1,3-β-glucanases) and protease inhibitors (Vigers *et al.*, 1991). By changing their physiological conditions, higher plants protect themselves from various stresses, such as pathogen attacks, wounding, and the application of chemicals including phytohormones and heavy metals, air pollutants like ozone, ultraviolet rays, and harsh growing conditions.

This reaction is known as the defense responses of higher plants, and a series of proteins actively synthesized with this reaction are called defense-related proteins (Bowles, 1990). Plant defense systems can be classified as either passive/ constitutive or active/ inducible, depending on whether they are pre-existing features of the plant, or are switched on after a challenge (Vigers *et al.*, 1991). Plant proteins that are induced as part of the plant defense response and purportedly function as antifungal agents by a mechanism involving interaction with the microbe that directly inhibits fungal growth or differentiation are termed antifungal proteins (Yun *et al.*, 1997). These include Pathogenesis- Related proteins, proteins first described by van Loon and van Kammen (Yun *et al.*, 1997) as components of the hypersensitive response in leaves of tobacco plants exposed to tobacco mosaic virus (TMV). PR proteins include all plant proteins that are induced in pathological or related situations. These proteins are now grouped into families based on primary structure, serological relatedness, and enzymatic and biological activities. This nomenclature defined five families of PR proteins based on protein categories that were identified in tobacco (Table 1.1, URL 5), but other PR proteins that cannot be categorized into one of the five major PR families have subsequently been characterised (Yun *et al.*, 1997).

Table 1.1. Recommended Classification of Pathogenesis-Related Proteins (PRs) (URL5)

Family	Type member	Properties
PR-1	tobacco PR-1a	antifungal, 14-17kD
PR-2	tobacco PR-2	class I, II, and III endo-beta-1,3-glucanases, 31-35kD
PR-3	tobacco P, Q	class I, II, and IV endochitinases, about 30kD
PR-4	tobacco R	antifungal, *win*-like proteins, endochitinase activity, similar to prohevein C-terminal domain, 13-15kD
PR-5	tobacco S	antifungal, thaumatin-like proteins, osmotins, zeamatins, permeatins, similar to alpha-amylase/trypsin inhibitors
PR-6	tomato inhibitor I	protease inhibitors, 6-13kD
PR-7	tomato P	endoproteases
PR-8	cucumber chitinase	class III chitinases, chitinase/lysozyme
PR-9	lignin-forming peroxidase	peroxidases, peroxidase-like proteins
PR-10	parsley PR-1	ribonucleases, Bet v 1-related proteins
PR-11	tobacco class V chitinase	endochitinase activity

1.7.1 Pathogenesis Related Proteins

PR proteins are induced by a variety of pathogens in a salicylate-dependant pathway and are thought to mediate a protective effect during SAR. Chemicals that can induce SAR also include PR proteins and have been shown to protect the plant against pathogen attack. Such protection has been achieved for example by

the application of benzothiadiazole against several biotrophic pathogens but not against nectrophic pathogens such as *Alternaria alternata* and *Botrytis cinerea*. Biotrophic fungi include the rust fungi and powdery mildews of the Basidiomycetes and Oomycetes respectively. These organisms usually cannot survive outside of the living host and use haustoria to obtain nutrients. Nectrophic fungi usually produce toxic substances which kill the plant cells and therefore live saprophytically on the dead tissue. SAR in radish and *Arabidopsis* can also be induced by biocontrol bacteria in the soil (Epple *et al.*, 1997).

1.7.1.1 PR-1

These proteins range in size from 15 to 17 kDa and are induced by salicylic acid. They are the most abundantly accumulated of the PR protein families after pathogen infection. Members of the PR-1 protein family from tobacco and tomato have been shown to have antifungal activities (Yun *et al.*, 1997).

1.7.1.2 PR-2 (β-1,3-Glucanases)

These are abundant proteins widely distributed in seed-plant species and have molecular masses ranging between 25 and 35 kDa. The majority of these proteins are endoglucanases, hydrolyzing oligomers of 2 to 6 glucose units from β-glucans. The β-1,3-glucanases accumulate during many physiological and developmental processes in addition to pathogen attack. *In vitro*, β-1,3-glucanase, in combination with chitinase inhibited fungal growth and act synergistically to degrade fungal cell walls, supporting their possible roles in defense against fungal pathogens *in vitro* (Dixon *et al.*, 1996). Transgenic tobacco plants expressing β-1,3-endoglucanase from soybean showed enhanced resistance to *Phytophthora parasitica var nicotianae*, an oomycete fungus (Dixon *et al.*, 1996).

1.7.1.3 PR-3 (Chitinase)

Chitinases are enzymes that hydrolyze the N-acetylglucosamine polymer, chitin. A direct role for chitinase in plant defense has been implied because there is no known substrate for these enzymes in plants, and chitin is a common component of many fungal cell walls (Dixon *et al.*, 1996). Chitinases can be detected at low levels but these levels increase in response to abiotic agents eg. ethylene, salicylic acid or ozone, and by biotic factors eg. fungi, bacteria, viruses, fungal cell wall components or oligosaccharides. Rapid and high level accumulation of chitinases occur both in tissues exhibiting a hypersensitive response, as well as those that are still susceptible to pathogen infection. The induction of chitinase is often coordinated with accumulation of specific β-1,3-glucanases and other PR proteins (Lawton *et al.*, 1992). Constitutive expression of a bean chitinase in transgenic tobacco and *Braccica napus* enhanced resistance against the fungal pathogen *Rhizoctonia solani*, causative agents of post-emergence damping off (Broglie *et al.*, 1991).

1.7.1.4 PR-4

These proteins are distinguishable from other PR proteins but are serologically similar to tomato protein P2 (Joosten *et al.*, 1990) and no function or enzymatic activity has been ascribed to these PR-4 proteins. A 20 kDa antifungal protein (CBP 20) was isolated from tobacco inoculated with TMV and using structure and immunological data, was shown to be a member of the PR-4 family (Yun *et al.*, 1997). Antifungal assays demonstrated that CBP 20 has antifungal properties against *Trichoderma viride* and *Fusarium solani* and act synergistically with a chitinase against *F. solani* and with a β-1,3-glucanase against *F. solani* and *Alternaria radicina* (Ponstein *et al.*, 1994).

1.7.1.5 PR-5 (Thaumatin-like Proteins)

This PR family includes osmotin, and homologs of osmotin including NP-24, a tomato osmotically regulated protein and tobacco TMV-induced proteins. Other homologs are a bifunctional α-amylase/trypsin inhibitor, and zeamatin from maize. Osmotin gene expression has been the most extensively characterized, and osmotin gene expression can be induced by several factors, including NaCl, dessication, ethylene, wounding and fungal infection (Kononowicz *et al.*, 1992). Several members of the PR-5 protein family have been shown to exhibit *in vitro* antifungal activities against a broad range of fungi (Vigers *et al.*, 1991).

In addition to PR proteins, there are others in plants that have antifungal activities. These include thionins, lipid transfer proteins, 2S storage albumin, ribosome- inactivating proteins, polygalacturonase inhibitors, nonenzymatic chitin-binding proteins and plant defensins (Yun *et al.*, 1997). Although not classically considered PR proteins, proteinase inhibitors are also induced by wounding, insect attack, and application of fungal elicitors or plant cell wall components (Vigers *et al.*, 1991). Several families of small, basic, cysteine-rich antimicrobial proteins may also play a role in plant defense. These include plant defensins (found in mammals and insects), lipid transfer proteins and thionins (found in leaves and seeds of different plants) (Epple *et al.*, 1997).

1.7.1.6 Thionins

The possible function of thionins has been debated for a long time. The toxic activity of purothionins was discovered soon after purification, and the antimicrobial activity of several thionins *in vitro* against phytopathogenic bacteria and fungi indicates a role in plant defense. Thionins are small proteins (5k Da), cysteine-rich and generally basic polypeptides, a characteristic of thionins thought to be important for toxicity. Another characteristic includes the presence of a tyrosine residue at position 13 in the peptide (Epple *et al.*, 1997). These proteins are synthesized as precursors in which the amino acid sequence of the

mature protein is preceded by a typical signal peptide and followed by an acidic carboxy terminal sequence (Herrera-Estrella and Simpson, 1995). They have been identified in a number of monocots and dicots, with those in barley being the most thoroughly characterized. Thionins were characterized into five well-defined structural type categories (Type I- Type V). Of these, Type II was determined to be toxic to phytopathogens *Thievaliopsis paradoxa*, a pathogen of sugar cane and *Drechslera teres*, a pathogen of barley. Combined with lipid transfer protein or 2S storage albumins, thionins evoke additive/ synergistic *in vitro* antifungal effects (Yun *et al.*, 1997). Furthermore, several thionin genes can be induced after pathogen attack. Epple *et al.* (1997) showed that the *Arabidopsis Thi* 2.1 gene is inducible by pathogenic fungi, and that this induction is faster and more intense in resistant ecotypes than in susceptible ecotypes. Thionins are thought to function by altering membrane permeability, inhibiting the biosynthesis of macromolecules and in participating in Redox reactions in the pathogen (Yun *et al.*, 1997).

1.7.1.7 Polygalacturonase Inhibitor Proteins

Proteinaceous inhibitors of fungal polygalacturonase have been defined and identified by their inhibition *in vitro* of fungal polygalacturonase activity and have been found in extracts of several plant tissues (Collmer and Keen, 1986). It is presumed that polygalacturonases function in pathogen infection by facilitating host cell wall degradation and PGIPs interfere with this process (Yun *et al.*, 1997). PGIPs from pear, tomato and bean have been characterized extensively and biochemical characterization of PGIPs has shown that they are relatively heat stable glycoproteins (Stotz *et al.*, 1993). Purified PGIPs from pear inhibit different fungal PG's but do not affect endogenous pear fruit PG activity, suggesting an interaction with exogenous PGs rather than those involved in ripening-related processes. These PGIPs from pear have been genetically engineered and constitutively expressed in tomato, with the fruits of the transgenic plants exhibiting reduced postharvest infection by *Botrytis cinerea* (Stotz *et al.*, 1993).

1.7.1.8 Lipid Transfer Proteins

Nonspecific lipid transfer proteins (ns LTPs) have been isolated from numerous plant species. Terras *et al.* (1995) purified a basic 9 kDa protein from radish (*Rhaphanus sativus*) seed that had *in vitro* antifungal activity against *Fusarium culmorum* and analysis of sequence data indicated sequence homology of the protein to nsLTPs. Two purified proteins (8.6 and 8.7 kDa) from the leaves of barley and an 8.6 kDa protein from maize showed 32 to 62% identity with other known members of the nsLTPs in plants. All of these proteins possessed growth inhibitory activity against *F. solani in vitro* (Yun *et al.*, 1997).

1.7.1.9 2S Storage Albumins

These proteins are generally considered storage proteins, but are known to inhibit the growth of pathogenic fungi. Terras *et al.* (1993) showed that a 2S albumin from *Brassicaceae* seeds inhibited fungal growth *in vitro*. They also showed that the radish 2S albumin synergistically enhanced the antifungal activity of thionin.

1.7.1.10 Ribosome Inactivating Proteins

Plant RIPs have greater specificity for non-host ribosomes, although these exhibit varying activities toward ribosomes of distantly related species, including fungi (Yun *et al.*, 1997). Leah *et al.* (1991) showed that barley RIP, in combination with chitinase or β-1,3-glucanase inhibited the growth of *Trichoderma Rees* and *Fusarium sporotrichioides*. Transgenic tobacco plants that expressed a barley RIP, individually, or combined with a chitinase exhibited increased protection against *Rhizoctonia solani* infection (Logemann *et al.*, 1992).

1.7.1.11 Non-Enzymatic Chitin- Binding Proteins

These are antifungal proteins that lack chitinase activity and include chitin-binding lectins from wheat, barley and stinging nettle, and a small protein from rubber tree latex called hevein (Broekaert *et al.*, 1992). All of these chitin-binding proteins share a homologous cysteine/glycine rich domain of about 40 to 43 amino acids. Antimicrobial peptides from *Amaranthus caudatus* seeds (*Ac*-AMP1 and *Ac*-AMP2) show sequence homology to the cysteine / glycine rich domain of chitin-binding proteins. *In vitro* experiments demonstrated antifungal activities of these proteins. Nettle lectin inhibited germ tube growth of seven fungi including *Botrytis cinerea*, *Colletotrichum lindemuthianum*, *Phoma betae*, *Phycomyces blakesleeanus*, *Septoria nodorum*, *Trichoderma hamatum* and *T. viride*. Hevein is on average 3 to 5 times less active than nettle lectin, although they have similar properties. The *Ac*-AMPs have the greatest antifungal activities inhibiting the growth of different plant pathogenic fungi at much lower doses than other known antifungal chitin-binding proteins and exhibiting antifungal activity against a much broader spectrum than nettle lectin or chitinase (Broekaert *et al.*, 1992).

1.7.1.12 Plant Defensins

These are the structural and functional homologues of insect and mammalian proteins that have well established roles in host defense. Fourteen members of plant defensins have been isolated from 13 different plant species. Several members of the plant defensin family inhibited growth of a broad range of filamentous fungi at micromolar concentrations. The significance of these proteins in seedling protection against pathogens was demonstrated when Terras *et al* (1995) allowed radish seeds to germinate (on a medium that supported fungal growth) and monitored the release of radish defensins (Rs-AFPs) during germination. The Rs-AFPs inhibited fungal growth.

1.7.2 Constitutive Antifungal Compounds

Biochemical defense mechanisms of higher plants for pathogenic fungi include the accumulation of antifungal metabolites in response to pathogen attack and the presence of constitutive antifungal compounds. The degradation of these substances to less toxic products is an important method used by pathogens to overcome host defenses (Friebe *et al.*, 1998).

1.7.2.1 Allelochemicals

These include cyclic hydroxamic acids and related benzoxazolinone compounds in gramineous plants. They are found in maize, rye and wheat, but not in rice, barley or oats, and are thought to be involved in plant disease resistance because of their inhibitory activity toward some fungi and bacteria. Antifungal effects on *Helminthosporium turcicum*, *Septoria nodorum* and *Microdoctium nivale* have been observed (Friebe *et al.*, 1998).

1.7.2.2 Phytoalexins

Phytoalexins are fungitoxic substances of low molecular weight acting against a wide variety of different pathogenic fungi. They are synthesized and accumulate in plants in response to microbial infection. PAs constitute a chemically heterogenous group of molecules typical of plant secondary metabolites which belong predominantly to the families of the phenylpropanoids, isoprenoids and acetylenes (Herrera-Estrella and Simpson, 1995). Different plant families produce different chemical classes of PAs. Several plants, including grapevine, synthesize the stilbene-type phytoalexin-resveratrol, when attacked by pathogens. Stilbenes with fungicidal potential are formed in several unrelated plant species such as peanut, grapevine and pine (Hain *et al.*, 1993). Isoflavonoids are found predominantly in the leguminosae, while solanaceae produce sesquiterpenes (Dixon *et al.*,1996). PAs are vitally important in certain pathogen resistance

interactions; in particular, virulence of *Nectria haematococca* on pea depends on its ability to degrade the hosts phytoalexin (Hain *et al.*, 1993).

1.8 Control of Pathogens

Control of pathogens by various means is centered mostly around the diseases caused by fungi, bacteria, nematodes and insects, and pest management has become a big issue in the world of agriculture. Chemical sprays were introduced in the 1970s and were shown to increase yields in 80% of the fields that they were applied in, but over time the pathogens began to build up resistance to certain chemicals. The pollution factor involved with spraying chemicals raised more concerns and newer approaches to pest management were implemented. These approaches included biological control (which is any means of controlling disease or reducing the effect of the pathogen that relies on biological mechanisms or other organisms) and cultural practices, but combining these approaches proved to be more effective than implementing them on their own. This is known as Integrated Pest Management (URL1).

1.8.1 Control of Fungal Pathogens

The majority of plant pathogenic fungi contain chitin and β-1,3 glucans in their cell walls, and dissolution or pertubation of these structural polymers has adverse effects on the growth and differentiation of fungi. Cell wall degrading enzymes, especially chitinolytic enzymes produced by biocontrol miro-organisms such as species of *Trichoderma* and *Gliocladium* are able to effectively control plant pathogenic fungi. . Combinations of cell wall degrading enzymes and fungicides may also act synergistically in the control of pathogenic fungi (Lorito *et al.*, 1994). Fungitoxic compounds in use include gliotoxin, a naturally occurring epipolythiogiketopiperazine produced by fungi such as *G. virens*; the triazole, flusilazole, which specifically inhibits a demethylation step in the synthesis of sterols in fungi, thereby affecting the fungal membrane structure and integrity, is widely used in agriculture; the imidazole miconazole, which has a similar mode of

action to that of flusilazole; benomyl; and captan, a plant protectant that binds to thiol groups and has a non-specific mode of action (Lorito *et al.*, 1994).

1.8.2 Control of Insect Pests

Chemical control of insect pests is expensive and constantly threatened by the ability of insects to develop resistance to chemical insecticides. Insects can be infected by disease-causing organisms such as bacteria, viruses and fungi, thus diseases can be important natural controls of some insect pests (URL 3).

1.8.2.1 Entomopathogenic Fungi

Some insect species, including many pests, are particularly susceptible to infection by naturally occurring insect-pathogenic fungi. These fungi are very specific to insects. The *Entomophthora* and related species are fairly specific with regard to the groups of insects affected; other fungi, such as *Beauveria*, have a wider host range. Because fungi penetrate the insect body, they can infect sucking insects such as aphids and whiteflies that are not susceptible to bacteria and viruses. Once inside the insect, the fungus rapidly multiplies throughout the body. Occasionally toxins are produced by the fungus, but death is usually caused by tissue destruction. Infected insects stop feeding and become lethargic. The effectiveness of entomopathogenic fungi against pests depends on having the correct fungal species strain with the susceptible insect life stage, at the appropriate humidity, soil texture and temperature. Common insect pathogenic fungi include *Entomophthora muscae*, *Pandora neoaphids*, *Zoophthora radicans*, *Beauveria bassiana*, *Metarhizium* spp, *Verticillium lecanii*, *Neozygites floridana* and *Hirsutella thompsonii*. These are pathogens of flies, aphids, diamondback moth, beetles and weevils, corn rootworm, greenhouse whitefly, spider mites and mites respectively. In some countries *Beauveria* is used as a fungal microbial insecticide, while in Europe *V. lecanii* is being used to combat greenhouse whitefly and aphids. Various strains of entomopathogens are commercially available to combat various insect pests (URL 3).

1.8.2.2 Plant Derived Enzyme Inhibitors and Lectins for Control of Insect Pests

1.8.2.2.1 α-Amylase Inhibitors

Most plant α -amylases have been described from leguminous and graminaceous crops. α-Amylases are the main carbohydrate-digesting enzymes in insect gut and plant resistance to pests is achieved presumably by interfering with carbohydrate assimilation from the insect diet (Burrows *et al.*, 1998). α-Amylases undergo proteolytic maturation and this process is necessary for the protein to become active. Proteolytic processing at an Asn-Ser site is required for the activation of α-amylase inhibitors, and this site is present in all of the α-amylase inhibitor sequences reported (Pueyo and Delgado-Salinas, 1997). α-Amylase inhibitors protect seeds of the common bean against predation by certain species of bruchids such as cowpea and azuki bean weevils (Ishimoto and Chrispeels, 1996) and transgenic peas expressing common bean α -amylase inhibitor were shown to be resistant to these bruchids. An α-mylase inhibitor from the seeds of *Amaranthus hybridus* (L.) strongly inhibits α-amylase activity in insect larvae (Burrows *et al.*, 1998).

Plant-derived α-amylase inhibitors tend to be strong inhibitors of α-amylase from animals, including a broad spectrum of insects or micro-organisms, but rarely against amylases from plants (Burrows *et al.*, 1998; Ryan, 1990). α-Amylase inhibitors, purified from wheat, were found to be potent inhibitors of amylases found in the midguts of the larvae of two storage pests of legumes- *Callosobruchus maculatus* and *Tribolium confusum* (Ryan, 1990).

1.8.2.2.2 Lectins

Lectins are proteins other than enzymes and antibodies that bind carbohydrate. They are widespread in nature and found in a variety of plant species. In plants, their physiological role is unknown, but evidence suggests that they are mainly involved in defense against pathogens and pests. Lectins from garlic and snowdrop are active against *Callosobruchus maculatus* and *Spodoptera litoralis*. Phytohaemagglutinin

(PHA) from *Phaseolus vulgaris* and wheat germ agglutinin (WGA) are toxic to the larvae of *Callosobruchus maculatus*. PHA is a lectin that consists of two different types of polypeptides, PHA-E and PHA-L, and binds to the glycoproteins of the intestinal mucosa of mammals and insects. The toxicity of another lectin- arcelin is related to its low digestibility. Arcelin is found in certain Mexican accessions of *P. vulgaris* that are resistant to the common bean weevils (Pueyo and Delgado-Salinas, 1997). The expression of lectin (P-LEC) at high levels in transgenic tobacco plants resulted in enhanced resistance to tobacco budworm, and an increase in resistance against insects was achieved with the expression of snowdrop lectin in tobacco plants (Burrows *et al.*, 1998).

1.8.2.2.3 Protease Inhibitors (PIs)

Protease Inhibitor proteins are among the defensive chemicals in plant tissues that are both developmentally regulated and induced in response to insect and pathogen attacks and these proteins (that form complexes with proteases and inhibit their proteolytic activity) are widespread in nature. Inhibitor families have been found that are specific for each of the four mechanistic classes of proteolytic enzymes, i.e. serine, cysteine, aspartic and metallo-proteases (Ryan, 1990). PIs are often found in high concentrations in seeds and other storage organs. They inhibit proteolytic enzymes from animals and microorganisms but interestingly, rarely inhibit proteolytic enzymes from plants. The plant PIs are generally small proteins having molecular weights under 50 000 Da and more commonly under 20 000 Da. A number of inhibitors from corn, potatoes and several legumes have minimum molecular weights of below 10 000 Da and are often present as dimers or tetramers. The inhibitors contain active sites for the inhibition of proteolytic enzymes that apparently endow them with their specificity. The trypsin-specific inhibitors always have either a Lys-X or Arg-X sequence at the binding site, whereas chymotrypsin-specific inhibitors usually have a Leu-X at their active sites. These sites are part of a very large binding area that is necessary for the proper structural alignments that confer the unusual stability to the enzyme-inhibitor complex. The emerging picture from structural and specificity

similarities among plant PIs from different sources indicates that the active inhibitor sites may have been conserved over millions of years of evolution and suggests that the inhibitory capacity is important for survival. It has been suggested that PIs may have important roles as regulators in controlling endogenous proteases; as storage proteins; and as protective agents directed against insect and/or microbial proteases (Ryan, 1973). But since the major proteases present in plants, used in processes such as protein mobilization in storage tissues, contain a cysteine residue as the catalytically active nucleophile in the enzyme active site, the case for serine protease inhibitors as defensive compounds against predators is clear-cut. Serine proteases are apparently not used by plants in large-scale protein digestion and thus the presence of large amounts of inhibitors with specificity toward these enzymes in plants cannot be for the purpose of regulation of endogenous protease activity (Gatehouse *et al.*, 2000)

1.9 Classes of Proteases

1.9.1 Cysteine Proteases

These proteases are common in animals, eukaryotic microorganisms and bacteria as well as in plants. Many plant species (pineapple, potato, corn, rice, cowpea and others) contain cysteine endopeptidases. They all appear to be homologues of papain, often with similar specificity. Cysteine proteases are not secreted as intestinal digestive enzymes in higher animals, but are found in the midguts of several families of Hemiptera and Coleoptera, where they appear to play important roles in the digestion of food proteins. These insects characteristically have mildly acidic pHs in their midguts near the optima pH of cysteine proteases (approximately pH5). Several members of the order Coleoptera are seed- and leaf- eating insects that are important pests of agricultural crops (Ryan, 1990). Almost all of the inhibitors of cysteine proteases are members of a family of inhibitors that are related by both structure and function, called cystatins. The interaction of cystatins with papain has been defined by X-ray crystallographic studies. These studies show that highly conserved residues and secondary structures in the amino acid sequences of cystatins, including

the cystatin "signature motif" QXVXG are part of the intimate contact regions between the inhibitor and target protease. A number of cysteine protease inhibitors from plants have been identified as cystatins including two inhibitors from rice seeds, inhibitors from corn, soybean and *Wisteria floribunda* seeds (Walsh and Strickland, 1993). Walsh and Strickland (1993) have shown that a potato papain inhibitor has eight papain binding sites per inhibitor molecule.

1.9.2 Aspartic and Metallo Proteases

In species of six families of Hemiptera, aspartic proteases were found along with cysteine proteases. Potato tubers possess an inhibitor of the aspartic protease, cathepsin D that exhibits considerable amino acid sequence homology with the trypsin inhibitor SBTI from soybeans. The inhibitors of the metallo-carboxypeptidases from tomato and potato tissues are polypeptides that strongly inhibit a broad spectrum of carboxypeptidases from yeast and plants (Ryan, 1990).

1.9.3 Serine Proteases

The serine proteases trypsin, chymotrypsin and elastase belong to a common protein superfamily and are responsible for the initial digestion of proteins in the guts of most higher animals. They are endopeptidases and *in vitro* are used to cleave long polypeptide chains, which are then acted on by exopeptidases to generate free amino acids. At least seven distinct families have been described in plants. They have a common mechanism of action, where the inhibitor binds to the active site on the enzyme to form a complex with a very low dissociation constant. A binding loop on the inhibitor, usually "locked" into conformation by a disulphide bond, projects from the surface of the molecule, and contains a peptide bond cleavable by the enzyme. This peptide bond may be cleaved in the enzyme-inhibitor complex, but cleavage does not affect the interaction (Gatehouse *et al.*, 2000).

1.10 Classes of Protease Inhibitors

Table 1.2 Families of plant protein protease inhibitors (Koiwa *et al.*, 1997)

Family	**Protease Inhibited**
Serine Protease Inhibitors	Trypsin and Chymotrypsin
Soybean trypsin inhibitor (Kunitz) family	
Bowman-Birk family	
Barley trypsin inhibitor family	
Potato inhibitor I family	
Potato inhibitor II family	
Squash inhibitor family	
Ragi I-2/maize trypsin inhibitor family	
Serpin family	
Cysteine protease inhibitors	Papain, cathepsin B,H,L
Metallo-protease inhibitors	Caboxypeptidase A,B
Aspartic protease inhibitors	Cathepsin D

Proteinaceous protease inhibitors are generally categorized according to the class of protease that they inhibit (Table 1.2). Some plant serine protease inhibitors are bifunctional, typically possessing trypsin and α-amylase inhibitor activities; other protease inhibitors exist as multidomain proteins in which each domain possesses functional PI activity (Koiwa *et al.*, 1997). The Kunitz and Bowman-Birk families of inhibitors are the most widely studied types of serine endoproteases inhibitors in plants. Both are typified by inhibitors isolated from soya bean seeds. Kunitz inhibitors are typically monomeric proteins with approximately 190

amino acid residues and two intra-chain disulphide bridges. Generally, distribution of Kunitz inhibitors seems to be sporadic across a range of plant families. Bowman-Birk inhibitors are common in seeds of legume species. They consist of polypeptides with about 70-80 amino acids and can form oligomers. The basic protein unit contains a high proportion of cysteine residues and forms multiple intra-chain disulphide bridges. These proteins are quite abundant in seeds. Another family of inhibitors from the seeds of barley and other cereals is the barley Trypsin Inhibitor family. These are proteins of M_r 11-14 000 and contain a single enzyme inhibitory site. They also contain a high cysteine content. Potato Inhibitors I and II families (PI-I and PI-II) are from potatoes and other members of the Solanaceae. They are based on relatively small polypeptides. PI-I has a low cysteine content and unusually for PIs, disulphide bonds are not essential for activity. Members of the Cucurbitaceae also contain a group of very small serine protease inhibitors in their seeds. The proteins consist of 29-32 amino acid residues but are relatively cysteine-rich (Gatehouse *et al.*, 2000).

1.11 Protease Inhibitors as Protective Agents

Protein digestion in animals is primarily accomplished extracellularly in the stomach, rumen or in the midgut of insects. Extracellular digestion of food is achieved by secretion of hydrolytic enzymes into the surrounding medium. When PIs are present in high concentration in foods of some farm animals, they can significantly alter digestive processes and interfere with growth and development. However, if high levels of PIs are present on a continual basis, it can activate complex feedback mechanisms and lead to chronic hypersecretion by the pancreas, loss of proteolytic activity in the gut, loss of appetite, starvation and eventual death. In insects, evidence indicates that feedback mechanisms that regulate digestive proteinase production in response to ingested foods containing proteinase inhibitor proteins are in place (Ryan, 1990). Insect damage to plant leaves can cause a striking increase in PI in various plants, apparently as a defense response of the plant. Attacks by pathogens and viruses can also induce synthesis of PIs (Ryan, 1990).

Wounding in species of Solanaceae, Fabaceae, Cucurbitaceae, Solicaceae and Poaceae induces systemic PIs (Giri *et al.*, 1998), and cysteine PI gene expression is also induced by wounding (Zhao *et al.*, 1996).

1.11.1 Protease Inhibitors and Insects

Studies on the effects of PIs on insect diets first began in the 1950s when Lipke *et al.* (1954) found that a protein fraction from soybeans inhibited growth, as well as proteolytic activity *in vitro*, of the meal worm *Tribolium confusum* (Ryan, 1990). All four classes of proteases have been identified as playing roles in protein digestion in different species within the insect kingdom, and inhibitors of all four classes of proteases have been identified in different plant species. Serine, cysteine and aspartyl proteases function as endopeptidases and are thus important in the initial stages of digestion, while metallo proteases are important as exopeptidases and function in secondary digestion (Gatehouse *et al.*, 2000). Plant serine proteases are known to affect the growth of herbivorous insects and may function as defensive agents against Lepidopteran pests such as *Helicoverpa* and *Spodoptera*, which use serine proteases to digest their food proteins (Giri *et al.*, 1998). It was assumed that serine proteases were the major digestive enzymes in all insects. However, many Coleopteran species and some Hemiptera, contain both cysteine and aspartyl proteases, but have little or no serine protease activity (Gatehouse *et al.*, 2000). *In vitro* assays show that plant cysteine PIs function in defense against insect predation, where they inhibit digestive cysteine proteases in insect guts. Bioassays provide evidence that these proteins possess insecticidal activities against Coleopteran insects (Zhao *et al.*, 1996). Transgenic plants with the gene for a PI from cowpea or potato showed increased resistance against attack of cotton budworm (*Heliothis virensis*) or tobacco hornworm (*Manduca sexta*) respectively. Transgenic rice plants containing a transformed gene for potato PI II showed increased resistance to the rice insect pest-pink stem borer (*Sesamia inferens*) in five generations (Dunaevsky *et al.*, 1998).

1.11.2 Protease Inhibitors and Fungi

Plants do not contain an immune system and must rely on other mechanisms to protect themselves from fungal infection. These mechanisms include the synthesis of inhibitory compounds such as phenols, melanins, tannins or phytoalexins as well as the accumulation of proteins (which include protease inhibitors) that can directly inhibit fungal growth (Chen *et al.*, 1999b). The most extensively studied protease inhibitors are the trypsin inhibitors, proteins of different size and amino acid sequence that can inhibit the activity of trypsin and some trypsin inhibitors have even been reported to be bifunctional inhibitors (Chen *et al.*, 1999b). However, the mode of action of many of these inhibitors against fungal pathogens has not been clearly demonstrated. Chen *et al.* (1999b) demonstrated the ability of a purified trypsin inhibitor (from corn kernels) to inhibit *in vitro* conidia germination and hyphal growth of *Aspergillus flavus*, while Dunaevsky *et al.* (1998) demonstrated the ability of protease inhibitors from buckwheat seed to suppress germination of spores of the filamentous fungi *Alternaria alternata* and *Fusarium oxysporum.* The PIs also inhibited proteases secreted by these fungi.

The characterization of the mode of action of protease inhibitors against fungi could constitute a first step leading to the development of selectable marker traits for plant breeders as well as the isolation of genes for use in developing resistant transgenic plants.

1.12 Transgenic Plants

The use of genes encoding plant protease inhibitors has been suggested as a means by which transgenic crops with enhanced insect resistance could be produced, and several examples of successful engineering of increased resistance by this means have been presented (Bown *et al.*, 1997). However, some insects can overcome the protease inhibitors by increasing proteolytic activity, by inducing different proteolytic enzymes that are insensitive to the corresponding plant protease inhibitors, or by expressing proteases that degrade protease inhibitors. In addition, some insect populations are genetically variable for tolerance to plant

protease inhibitors. For instance, when populations of the weevil *Geutorynchus assimilis* were collected from two oilseed rape fields in France and reared on transgenic oilseed rape expressing a rice cysteine protease inhibitor, one strain showed an increase in growth rate whereas the other remained unaffected (Stotz *et al.*, 1999).

Chapter 2

Methods

Chapter 2
Methods

Introduction

In plants, protease inhibitor (PI) families specific for each of the four mechanistic classes of proteases have been identified. PIs have been isolated from bitter gourd, different species of Erythrina, beans, potato and soybean (Dunaevsky *et al.*, 1998) as well as from other species in Leguminosae, Gramineae and Solanaceae (Broadway, 1993). In order to study these PIs and determine their functional roles, preparations of individual inhibitors are needed. PIs have been isolated by extraction in various buffers, glycogen or ammonium sulphate precipitation and purified from plant tissues by the use of a variety of techniques which include affinity and ion-exchange chromatography and HPLC.

2.1 Extraction

The procedure for the extraction of protease inhibitors from wheat, *Triticum aestivum* cv. Witwol seeds was modified from that described by Dunaevsky *et al.* (1998). Wheat seeds were obtained from farmers in the Steinkopft area, Namaqualand during 1999 and 2000.

The seeds (500g) were milled to a fine powder prior to extraction. This served as the starting material which was extracted in Buffer A- 0.1M Sodium phoshate buffer, (pH6.8) (1:4 w/v) by stirring the mixture for 16 hrs at 4^0C. The extract, designated the crude wheat extract was centrifuged at 10 000rpm (Beckman Model J2-21 M Induction drive Centrifuge, JA 14 rotor) for 40 min at 4^0C. The resulting supernatant was stirred continuously on ice and brought to 80% saturation by slowly adding finely ground ammonium sulfate, $(NH_4)_2\ SO_4$ This ammonium sulfate supernatant was stirred at 4^0C, overnight. After centrifugation at 9000 rpm (Beckman Model J2-21 M Induction drive Centrifuge, JA 14 rotor) for 15 min at 4^0C, the resulting pellet was carefully resuspended in 150ml Buffer A, and dialysed against several changes of the same buffer at 4^0C using dialysis tubing with a molecular

weight cut-off of 6-8000 until the pH of the dialysate reached pH6.8. The dialysate was stored at 4^0C and used in subsequent chromatography steps, protein concentration determination and enzyme inhibitory activity assays.

2.2 Purification

Affinity chromatography provides opportunities for the isolation of substances according to their biological function since the bed material (an insoluble matrix) has biological affinity for the substance to be isolated. This affinity is achieved by coupling the appropriate binding ligand to the insoluble matrix. The ligand is able to adsorb the substance to be isolated from solution, and as experimental conditions are changed (after unbound substances are washed away), the binding ligand is then able to de-adsorb the isolated substance. Beaded agarose is used as a matrix because of its relative stability, biological inertness and superior chromatographic properties

Sepharose 4B displays virtually all the desirable features of a matrix for the immobilization of biologically active materials, and CNBr activated Sepharose 4B has been widely used for the immobilization of proteins because of the gentle conditions employed to obtain stable attachment.

Affinity chromatography was employed to separate the PIs isolated from wheat seeds from other contaminating proteins.

2.2.1 Preparation of Resin for Affinity Columns

Activated CNBr Sepharose 4B (Pharmacia), as supplied by the manufacturer, was used for affinity chromatography and prepared according to the guidelines in the instruction manual with either Subtilisin Carlsberg (Sigma) or Bovine Trypsin (Sigma) as the ligand.

Approximately 12 ml of the gel suspension was prepared as follows: 4g of the activated CNBr Sepharose 4B beads were prepared by washing with 800ml 1mM HCl on a sintered glass filter. 60mg of the ligand was coupled to the beads by dissolving the ligand in 25ml coupling buffer (0.1M Na HCO_3 (pH8) containing 0.5M NaCl), and adding this ligand solution to the washed gel suspension.

The final ratio of beads to ligand solution was 1:2. To facilitate coupling of the ligand to active sites on the Sepharose beads, the mixture of resin and ligand was thoroughly mixed and allowed to rotate end-over-end at 4^0C for 4 hrs. Excess ligand was washed away with five gel volumes of coupling buffer, and any remaining active groups blocked by adding 50ml 0.1M Tris-HCl (pH8) and allowed to stand for 1 hour at 4^0C. Finally, the ligand-bound resin was washed with 3 cycles of alternating pH buffers. Each cycle consisted of a wash with 10ml Sodium acetate buffer (pH4) containing 0.5M NaCl, followed by a wash with 10ml 0.1M Tris-HCl buffer (pH8) containing 0.5M NaCl. The resin was stored in 0.1M Tris-HCl buffer (pH8) containing 0.5M NaCl at 4^0C. The subtilisin-bound Sepharose 4B was used in batchwise purification of PIs, while the trypsin-bound Sepharose 4B was packed into a column.

2.2.2 Preparation of Ion-Exchange Columns

Ion-Exchange Chromatography was performed using a BioCAD/SPRINT (PerSeptive Biosystems). The procedures employed were modified from those described by Dunaevsky *et al.* (1998).

2.2.2.1 Cation-Exchange Chromatography

SelfPack POROS 20 HS was used in a column with dimensions 0.46 x 10cm (PerSeptive Biosystems) and equilibrated with 0.1M Sodium acetate buffer (pH4) prior to sample application. All samples were injected into the sample loop manually. The column was developed with a stepwise pH gradient of 0.1M Tris-HCl buffer (pH8) and a stepwise gradient of 0.1M Tris-HCl buffer (pH8) containing 1M and 2M NaCl respectively at a flow rate of 10ml/min. Fractions were eluted at a rate of 10ml/min and monitored by UV absorbance at 280nm for protein content. Fractions were stored at 4^0C before being assayed for inhibitory activity.

2.2.2.2 Anion-Exchange Chromatography

SelfPack POROS 20 HQ was used in a column with dimensions 0.46 x 10cm (PerSeptive Biosystems) and equilibrated with 0.1M Citrate-acetate buffer (pH4) prior to sample application. All samples were injected into the sample loop manually. The column was developed with a stepwise salt gradient of 0.1M Citrate-acetate buffer (pH4) containing 2M NaCl and a pH gradient of 0.1M Tris-HCl buffer (pH8) at a flow rate of 10ml/min. Fractions were eluted at a rate of 1ml/min and monitored by UV absorbance at 280nm for protein content. Fractions were stored at 4^0C before being analysed by mass spectroscopy.

2.2.3 HPLC

Inhibitors were purified by reverse-phase HPLC on a Vydac C18 analytical column with a solvent gradient of 100% 0.1% (v/v) TFA in distilled water (Solvent A) to 100% 0.1% (v/v) TFA in 100% acetonitrile (Solvent B) over 85 minutes at a flow rate of 1ml/min. Eluted protein peaks were monitored by measuring absorbance of the eluent at 230nm.

2.3 Assay Methods

2.3.1 Protein Determination: Bradford Assay

Protein concentration of all fractions was determined according to the Bradford method (Bradford, 1976) using Bovine Serum Albumin (BSA) (Sigma) as a protein standard. BSA was used to construct a standard curve from 0 to 0.6mg protein/ml (Figure 2.1).

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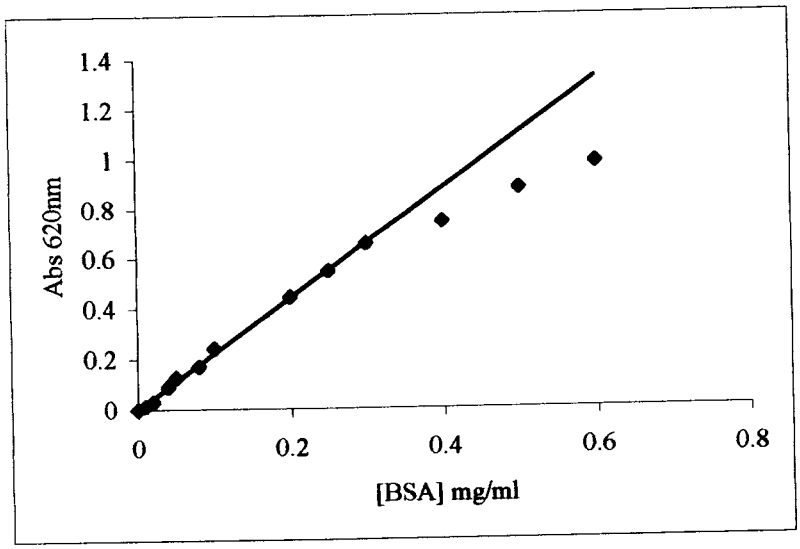


Figure 2.1 BSA Standard Curve using BSA at concentrations 0mg/ml to 0.6mg protein/ml. All assays were performed in duplicate and the average values used for all plots. All protein determinations were performed in the linear range 0 to 0.3mg/ml (R^2=0.9979)

Procedure:

20µl of a BSA standard was added to 200µl of Bradford Reagent per well in a microtitre plate. Absorbance was recorded at 620nm on a Multiskan® BICHROMATIC (Labsystems) plate reader and a standard curve constructed. Where samples were being assayed for protein concentration, 20µl of the sample was added to 200µl of Bradford Reagent. Appropriate blanks consisting of the relevant buffers were used and all assays were performed in duplicate.

2.3.2 Enzyme Assays

The serine endoproteinases trypsin, chymotrypsin and elastase are responsible for the initial digestion of proteins in the gut of most higher animals. *In vivo* they are used to cleave long polypeptide chains, which in turn are acted on by exopeptidases to generate amino acids, the end-point of protein digestion. Trypsin cleaves C-terminal to residues carrying a basic side-chain i.e. lysine and arginine, while chymotrypsin cleaves C-terminal to residues carrying a hydrophobic side chain i.e phenylalanine, tyrosine and leucine (Gatehouse *et al.*, 2000).

Serine PIs are widely distributed in the plant kingdom and inhibit the activity of those enzymes that are commonly responsible for digestion in animals and microorganisms (Broadway, 1993), but inhibitors that specifically inhibit proteolytic enzymes from microorganisms and not digestive proteases of animals are also common in plants, especially legume seeds (Ryan, 1990). The inhibitory potential of PIs isolated from wheat against commercial trypsin and chymotrypsin was determined using the assays described by Schwert and Takenaka (1955) and Hummel (1959), respectively.

2.3.2.1 Tryptic Assays

2.3.2.1.1 Amidolytic Activity of Trypsin using BAPNA as Substrate

Amidolytic activity of trypsin was measured using the synthetic substrate BAPNA- HCl (MW 434.9). Upon hydrolysis of the arginine-nitroanilide bond a yellow colour is produced which can be followed spectrophotometrically at 410nm. The assay buffer consisted of 50mM Tris-HCl (pH8.2) containing 10mM $CaCl_2$. The substrate stock solution, with a final concentration of 1mM, was prepared by dissolving 8.7mg BAPNA in 1.2ml DMSO and made up to 20ml with assay buffer. Progress curves of varying trypsin concentrations (2.5, 5, 10, 20, 40 and 50 µg per assay) were constructed (Figure 2.2).

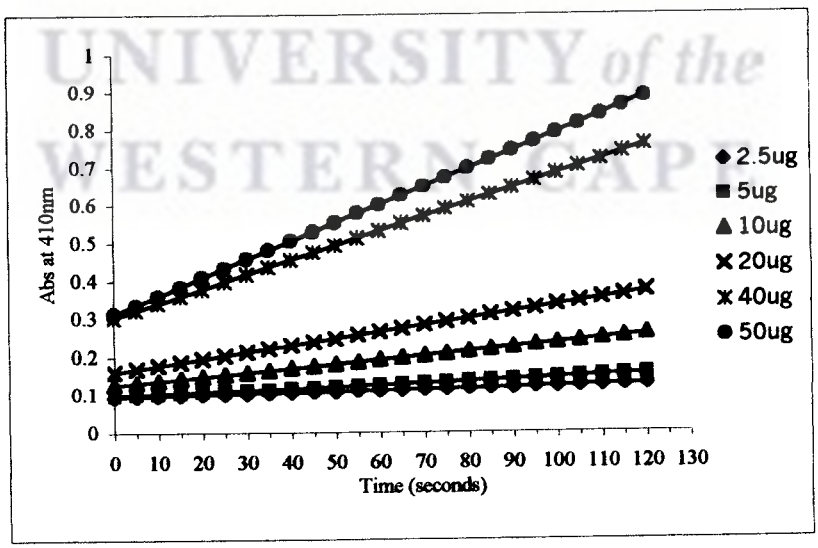


Figure 2.2 Trypsin progress curves using BAPNA as substrate and trypsin concentrations of 2.5, 5, 10, 20, 40 and 50 µg per assay. All assays were performed in triplicate and the average values used for all plots.

Procedure:

For each enzyme concentration being assayed, 900µl of substrate solution was added to a clean cuvette. 50µl of trypsin solution and 50µl dH_20 was added to the substrate solution and mixed. The change in absorbance at 410nm (using a Spectronic® GENESYS 5 spectrophotometer) was recorded at 5 second intervals for 2 minutes at room temperature.

Appropriate blanks (1ml of the assay buffer) were used and all assays were performed in triplicate.

From the linear portions of the progress curves, activity values (delta A410nm/min) were calculated and plotted against trypsin concentration. These values were used to construct a BAPNA standard curve (Figure 2.3).

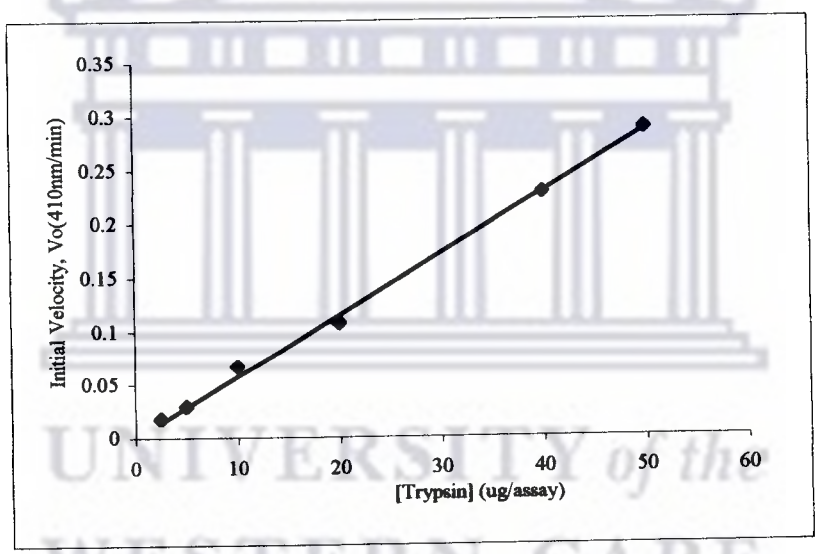


Figure 2.3 BAPNA Standard Curve using trypsin at concentrations of 2.5, 5, 10, 20, 40 and 50 µg per assay (R^2=0.9974)

2.3.2.1.2 Esterolytic Activity of Trypsin using TAME (MW 378.9) as substrate

Esteroytic activity of trypsin was measured using the synthetic substrate TAME. Upon hydrolysis of the arginine-methyl or lysine-methyl ester bond a product is produced which could be followed spectrophotometrically at 247nm. The assay buffer consisted of 50mM Tris-HCl (pH8.2) containing 10mM$CaCl_2$. The substrate stock solution with a final concentration of 1mM, was prepared by

dissolving 9.47 mg TAME in 25ml Assay buffer. Progress curves of varying trypsin concentrations (0.5; 1; 2; 4; and 5µg per assay) were constructed (Figure 2.4).

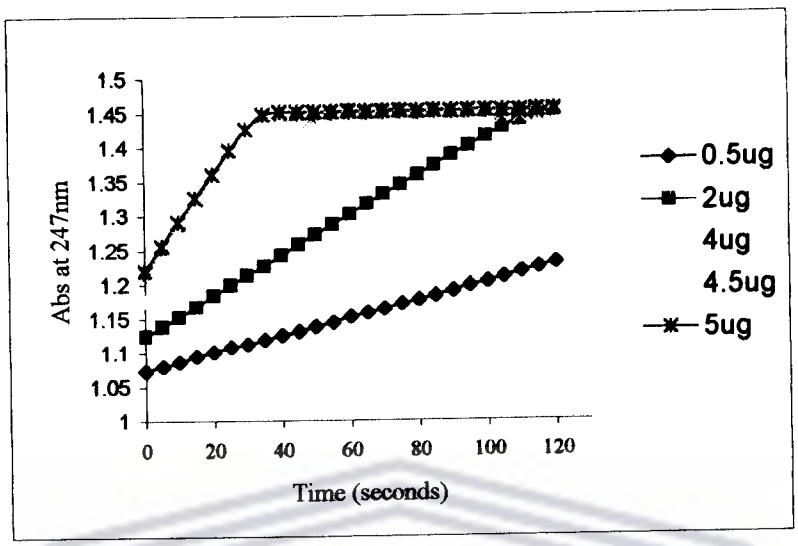


Figure 2.4 Trypsin progress curves using TAME as substrate and trypsin concentrations of 0.5, 1, 2, 4, and 5µg per assay.

Procedure:

For each enzyme concentration being assayed, 900µl of substrate solution was added to a clean cuvette. 50µl of each trypsin solution and 50µl dH_20 was added to the substrate solution and mixed. The change in absorbance at 247nm was recorded at 5 second intervals for 2 minutes at room temperature. Appropriate blanks (1ml of the assay buffer) were used and all assays were performed in triplicate.

From the linear portions of the progress curves, activity values (delta Abs247nm/min) were calculated and plotted against trypsin concentration. These values were used to construct a TAME standard curve (Figure 2.5).

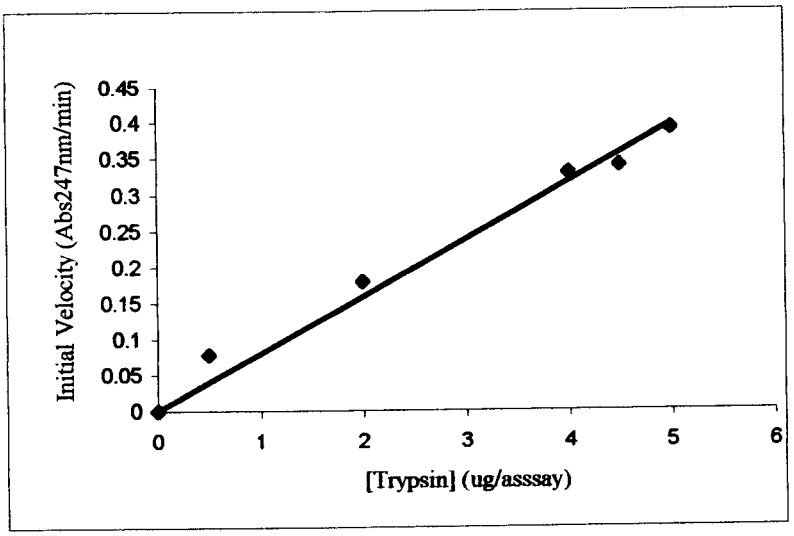


Figure 2.5 TAME Standard Curve using trypsin at concentrations of 0.5, 1, 2, 4, and 5µg per assay (R^2= 0.9807)

2.3.2.2. Chymotryptic Assay

2.3.2.2.1 Esterolytic Activity of Chymotrypsin using ATEE (MW 251.3) as substrate

Esterase activity of chymotrypsin was measured using the synthetic substrate ATEE. The assay buffer consisted of 50mM Tris-HCl (pH8.2) containing 10mM $CaCl_2$. The substrate stock solution, with a final concentration of 1mM, was prepared by dissolving 5mg of substrate in 20ml assay buffer. Progress curves of varying chymotrypsin concentrations (3, 4, 4.5, and 5µg per assay) were constructed (Figure 2.6).

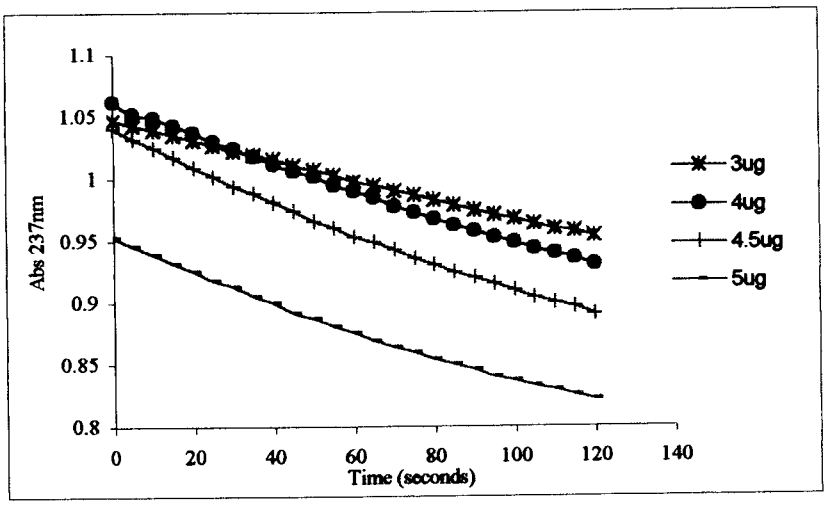


Figure 2.6 Chymotrypsin progress curves using ATEE as substrate and chymotrypsin at concentrations of 3, 4, 4.5 and 5µg per assay. All assays were performed in triplicate and the average values used for all plots

Procedure:

For each enzyme concentration being assayed, 900µl of substrate solution was added to a clean cuvette. 50µl of each chymotrypsin concentration and 50µl dH_20 was added to the substrate solution and mixed. The change in absorbance at 237nm was recorded at 5 second intervals for 2 minutes at room temperature. Appropriate blanks (1ml of the assay buffer) were used and all assays were performed in triplicate.

From the linear portions of the progress curves, activity values (delta A237nm/min) were calculated and plotted against chymotrypsin concentration. These values were used to construct an ATEE standard curve (Figure 2.7).

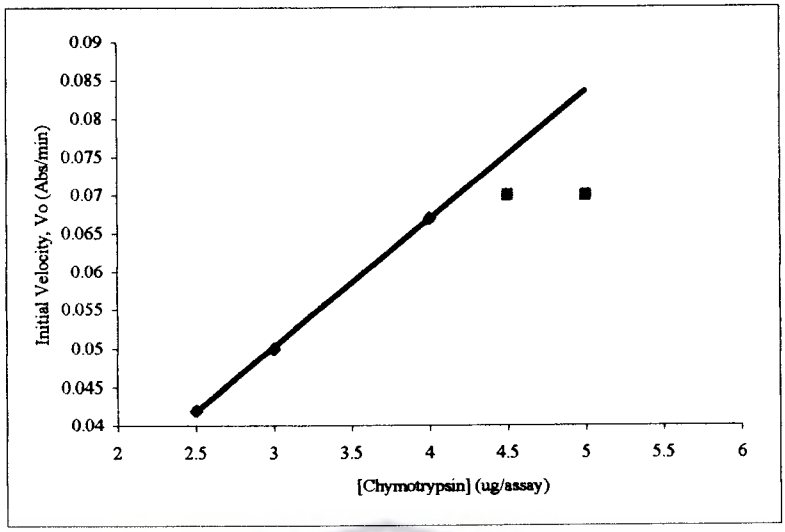


Figure 2.7 ATEE Standard Curve using chymotrypsin at concentrations of 3, 4, 4.5, and 5µg per assay All chymotrypsin assays were performed in the linear range 2.5 to 4ug chymotrypsin per assay (R^2= 0.9998)

2.3.2.3 Inhibitory Activity of Protease Inhibitors Against Trypsin

Trypsin inhibitory activity of the isolated PIs was determined by measuring the inhibition of amidolytic (using BAPNA as substrate) and esterolytic activity (using TAME as substrate). 50µg of trypsin per assay and 2µg trypsin per assay was used for assays with BAPNA and TAME respectively. The assay buffer used was the same as that used for the tryptic assays described in section 2.3.2.1.

Inhibitory activity was assayed as follows:

50µl of trypsin was added to 50µl of the inhibitor. This was incubated at room temperature for 1min, after which 900µl of the substrate was added. Absorbance at 410nm (for BAPNA) and 247nm (for TAME) was recorded at 5 second intervals for 2 minutes. The control reaction was performed as described above, except that the inhibitor was replaced by 50µl dH_20. Appropriate blanks (1ml of the assay buffer) were used and all assays were performed in triplicate.

2.3.2.4 Inhibitory Activity of Protease Inhibitors Against Chymotrypsin

The inhibitory activity of PIs against chymotrypsin was determined in the same way as described for inhibitory activity against trypsin, with the exception that ATEE was used as the substrate and that chymotrypsin was used at a concentration of 5µg per assay. Absorbance at 237nm was recorded at 5 second intervals for 2 minutes.

2.3.2.5 Protease Inhibitors and Insect Gut proteases

There is heavy reliance among insects on endoproteinases for the purpose of assimilating dietary protein. Insects having a midgut pH in the neutral or alkaline range generally rely on serine proteinases that are trypsin-, chymotrypsin-, or elastase-like, based on their similarity to mammalian enzymes. In contrast, insects having acidic or mildly acidic midguts generally utilize cysteine proteinases, such as cathepsins B, H, and L and/or aspartyl proteinases such as cathepsin D and pepsin (Wilhite *et al.*, 2000). PIs are believed to have a role in the natural defense of plants against insect pests, and the ability of PIs to interfere with insect growth and development has been attributed to their capacity to bind to, and thereby inhibit the action of insect digestive proteinases (Wilhite *et al.*, 2000).

Helicoverpa armigera (Lepidoptera: Noctuidae), the bollworm or ear worm, has a widespread distribution in tropical, subtropical and warm temperate regions, and is an important pest of many crop plants. They have proved especially difficult to control by conventional methods due to the build-up of resistance to pesticides within the population. Proteolytic activities in the gut of *H. armigera* have been investigated and shown to be due largely to extracellular serine proteases with high pH optima. *H. armigera* was found to possess trypsin-like activity, and inhibition of the proteolytic activity has been demonstrated *in vitro* using plant PIs, of which Soybean Kunitz trypsin inhibitor (SKTI) exhibited the greatest levels of inhibition (Bown *et al.*, 1997).

2.3.2.5.1 Inhibitory Activity of PIs Against Insect Gut Proteases

The inhibitory activity of the isolated PIs against insect gut proteases was determined by measuring the inhibition of serine protease activity in *Helicoverpa armigera* gut extract. This was done using the fluorescent substrate Z-Arginine-Amino-Methyl Coumarin (Z-Arg-AMC) as described by Bown *et al.* (1997).

Preparation of *H.armigera* Gut Extract

H. armigera gut extract was supplied by Dr. D. Bown, Department of Biological Sciences, University of Durham. The extract was prepared as follows: larvae were chilled on ice, decapitated and the midguts and contents dissected over ice. This material was immediately homogenized in dH_2O, aliquoted and frozen at -20^0C until required. After thawing, the aliquots of gut homogenate were made 1mM with respect to DTT, vortexed with 4 volumes of chloroform and centrifuged at 12 000g for 5 minutes at room temperature. The aqueous fraction was removed and retained for use in assays (Bown *et al.*, 1997). The assay buffer consisted of 0.5M Borate buffer (pH 9.8). The substrate (Z-Arg-AMC) was made up to a final concentration of 1mM with respect to DTT. The total volume of the reaction was 100µl. The volume of gut extract, borate buffer and substrate added to each reaction well remained constant. To determine the effect of the PIs on the gut extract, different volumes of inhibitor were used. Where dilutions were required, these were prepared using sterile distilled water. Soybean Kunitz trypsin inhibitor (SKTI) was used as a standard.

Activity was assayed as follows:

10µl of the *H. armigera* gut extract, 20µl borate buffer, 10µl inhibitor and 50µl dH_2O were added to sterile eppendorf tubes and pre- incubated in a water bath at 27^0C for 5 minutes. This reaction mixture was added to 10µl of the substrate (1mM Z-Arg-AMC) in a microtitre plate. Fluorescence of Z-Arg-AMC was recorded at 20 second intervals over a 10 minute period in a fluorimeter, where the filter pair wavelengths were 355nm excitation and 460nm emission. All assays were performed in triplicate.

2.4 Kinetic Parameters

Inhibition by protease inhibitor proteins involves the formation of a stoichiometric enzyme-inhibitor complex, which resembles a Michaelis complex. However, the energy barrier for hydrolysis is large and unfavourable, resulting in low rates of hydrolysis. In most cases the inhibition is mediated by an exposed loop that is fixed in a characteristic "canonical" conformation, which fits into the active site of the protease in a manner thought to be similar to that of a substrate. This loop is frequently constrained by the presence of disulfide bridges and /or extensive hydrogen-bonding networks that act to lock the structure into the correct canonical structure. The sequence of this loop determines the specificity of inhibitors, which mirrors the specificity of proteases for their substrates (Gariani *et al.*, 1999).

The inhibition kinetics of the inhibitor measured against bovine trypsin (Sigma) was determined using the substrates BAPNA and TAME at 3 different substrate concentrations (0.5mM; 0.75mM and 1mM) and 4 different inhibitor concentrations (ranging from 3.6µg to 18µg). Trypsin concentrations used were 50µg per assay for amidolytic activity using BAPNA as substrate and 2µg per assay for esterolytic activity using TAME as substrate. All experiments were performed at room temperature in assay buffer (50mM Tris-HCl (pH8.2) containing 10mM $CaCl_2$) and monitored through the change in absorbance at 410nm and 247nm for BAPNA and TAME, respectively as described in section 2.3.2.1.

The mechanism of inhibition was established by constructing Lineweaver-Burke plots, in which the inverse of the initial rate (1/V) is plotted against the inverse of the substrate concentration (1/[S]) in the presence of inhibitor. The dissociation equilibrium constant of inhibitor binding (Ki) is determined by constructing Dixon plots, in which the inverse of the initial rate (1/V) is plotted against the concentration of inhibitor ([I]) used at different substrate concentrations, or constructing Cornish-Bowden plots, where V/[S] vs [I] is plotted.

2.5 Physical Characterization

2.5.1 Electrophoresis

The molecular weight of isolated protease inhibitors was estimated by discontinuous SDS-PAGE, based on the method described by Laemmli (1970) using a BioRad mini III electrophoresis unit. Molecular weight markers used ranged in size from 94 000 to 14 000.

Procedure:

20µl sample buffer (previously reduced by the addition of 50µl β-mercaptoethanol to 0.95ml stock sample buffer) was added to 10µl of the protein sample to be analysed. Samples were heated at 95°C for 5 minutes in a water bath and loaded onto a 4% stacking, 15% resolving gel. Gels were electrophoresed at a constant 200V for 45 minutes.

Coomassie Blue Staining

Gels were stained using 0.1% Coomassie Blue (w/v) in 45% methanol and 10% acetic acid for 30 min with constant shaking. Destaining was performed in a 25% methanol, 10% acetic acid solution (v/v). Gels were destained overnight.

2.5.2 Mass Spectroscopy

HPLC purified proteins were analysed on a MALDI TOF mass spectrometer (Voyager-DE Biospectrometry Workstation, PerSeptive Biosystems) to determine the molecular mass of isolated proteins. In Matrix-Assisted Laser Desorption Ionization (MALDI), the sample is embedded in a low molecular weight, UV absorbing matrix that enhances sample ionisation. Time–Of–Flight mass spectrometry works on the principle that if ions are accelerated with the same potential from a fixed point and at a fixed initial time and are allowed to drift, the ions will separate according to their mass-

to-charge ratios. Lighter ions drift more quickly to the detector, heavier ions drift more slowly. The MALDI-MS was fitted with a nitrogen UV laser (337nm), and the matrix used was Sinapinic Acid (10mg/ml) with 50% acetonitrile, 3% TFA in deionised water as solvents. Analysis was performed by Prof. W. Brandt, Dept. of Biochemistry, UCT as per manufacturers instruction manual.

2.5.3 Trypsin Digest

The possible identity of purified PIs was determined by In gel tryptic digestion (performed by Prof. W. Brandt, Dept. of Biochemistry, UCT) as described at URL 6, followed by mass spec analysis to obtain a molecular weight profile. Candidate proteins were identified using MS-Fit software (URL 7).

Procedure:

1. Prewash 3 x 1.5 ml Eppendorf tubes with 500 µl 0.1% TFA/60% CH_3CN
2. Put the Coomassie Blue stained gel band, which should be cut into small pieces, into one prewashed tube
3. Remove one lane of a transferrin control (supplied by Keck Facility) containing 25 pmol and put in a separate prewashed 1.5 ml tube
4. Also take a blank section of gel (supplied by investigator-user) that should not contain protein and put in a prewashed 1.5 ml tube
5. Add 250µl 50% H_2O/ 50% acetonitrile and wash for 5 minutes
6. Remove wash
7. Add 250µl 50% CH_3CN/ 50 mM NH_4HCO_3 to all samples
8. Wash for 30 minutes at room temp on tilt table
9. Remove wash
10. Add 250µl 50% CH_3CN/10 mM NH_4HCO_3 to the gel pieces
11. Wash for 30 minutes at room temperature on a tilt table
12. Remove wash

13. Speedvac gel pieces to COMPLETE dryness

14. Add 0.1µg modified trypsin (Promega) per 15 mm^3 of gel in 15µl 10 mM NH_4HCO_3 to all samples and the blank

15. Let stand for 5-10 minutes to allow enzyme/buffer solution to absorb into the gel

16. Add an additional 20µl 10 mM NH_4HCO_3 that does not contain enzyme

17. Incubate at 37^0C for 24 hours

18. Hold all samples in the refrigerator pending MALDI-MS protein identification

19. For MALDI-MS take 1µl of each supernate and mix with 1µl of internal standards (containing 100 fmol each of two standards) and 1µl-cyano matrix (4.5 mg/ml) and then subject to MALDI-MS for peptide mass searching. Store the remaining gel in the refrigerator while the MALDI-MS is in progress

20. If peptide mass searching does not identify the sample, extract the peptides by adding 200µl 0.1% TFA, 60% CH_3CN and shaking at room temperature for 60 min (use more volume if necessary)

21. Remove wash containing peptides and add to a 1.5 ml Eppendorf prewashed (i.e. see step 1) tube

22. Repeat step 20 and 21

23. Speedvac dry the combined washes

24. Samples destined for LC/MS/MS protein identification on the LCQ should be dissolved in 60µl 0.05% TFA, 5% acetonitrile while samples destined for preparative HPLC should be dissolved in 45µl of the same solvent

25. Filter all samples; for LC/MS/MC go to step 26, for preparative HPLC go to step 28

26. Load 14µl of the filtered extract onto the LC/LCQ mass spectrometer which will then inject 10/60µl or 16.7% of the original digest

27 To collect the remaining digest (assuming that 16.7% had been subjected to LC/LCQ mass spectrometry and had screened and not been identified), transfer the remaining 46µl of the digest into the HP1090 sample vial, rinse the Eppendorf tube that contained the digest with 10µl 100% TFA and add to the HP1090 sample vial and vortex. The HP1090 will then inject 50µl or 50/56 x 46/60 = 68% of the original digest

28. To collect the remaining digest (assuming that no LC/LCQ analysis was carried out) transfer the remaining 45µl of the digest into the HP1090 sample vial, rinse the eppendorf tube that contained the digest with 10µl 100% TFA and add to the HP1090 sample vial and vortex. The HP1090 will then inject 50µl or 50/55 = 91% of the original digest - not taking into account the MALDI screen

29. Carry out reverse phase HPLC using a Vydac C18 (10 x 250 mm) column run at 30µl/min

2.5.4 MS-Fit

MS-Fit is a peptide mass fingerprinting tool from the UCSF Mass Spectrometry Facility that tries to fit a user's mass spectrometry data to a protein sequence in an existing database and thus suggest the identity of the user's protein. The MS input data should be generated by analyzing the peptides produced by the enzymatic digestion of a user's protein (URL 7).

2.5.5 CNBr Cleavage

CNBr digestion of proteins was performed by Prof. W. Brandt, Dept. of Biochemistry, UCT) as described by Gross (1967).

2.6 Cloning and Expression

Currently, crop protection relies primarily on synthetic chemical pesticides. However, this chemical approach to crop protection is coming under increasing pressure. The use of genes encoding plant PIs has been suggested as a means by which transgenic crops with enhanced insect resistance could be produced, and several examples of the successful engineering of increased resistance by this means have been presented (Bown *et al.*, 1997).

2.6.1 Amplification of *T.aestivum* α-amylase/trypsin inhibitor (P16159)

The oligonucleotide primers TI 5′ NdeI: cat atg atc ggc aat gaa gat tgc acc cca tgg atg

and TI 3′ XhoI: ctc gag gct cca ctg aga ctc ctc cat agc gag were synthesized from the regions flanking the mature wheat α-amylase/trypsin inhibitor (P16159) coding sequence. NdeI and XhoI restriction sites were incorporated into the 5' and 3' primers respectively to facilitate cloning into a pET expression vector. The P16159 gene was amplified by PCR from an EST clone (WHE 0059_D03_G05) obtained from Dr. Anderson, USDA, California. The PCR reaction (50µl) was set up as follows: DNA (5µl wheat EST clone), 10X Reaction Buffer (5µl), dNTPs (3µl 10 mM), 5' Primer (2µl TI Nde I), 3' Primer (2µl TI Xho I), Taq Polymerase (1µl), $MgCl_2$ (2µl 50mM), dH_2O (30µl) and amplified as follows: 94^0C 5 min (1 cycle), 94^0C 30 seconds, 50^0C 30 seconds, 72^0C 1.5 min (15 cycles), 72^0C 7min, 4^0C 1min (1 cycle).

2.6.2 Cloning into pCR 2.1

The reaction mixture (a total volume of 5µl) consisted of vector (1µl pCR® 2.1 TOPO vector), PCR product (3µl), dH_2O (1µl), was set up in an eppendorf tube and left at room temperature for 20 minutes.

2.6.2.1 Transformation

2µl of the plasmid mix was added to 50µl TOPO® TOP 10 F´ cells (or 50µl DHα5 competent cells). This was placed on ice for 30 minutes. The cells were then heat shocked by placing the tube in a water bath at 42^{0}C for 45 seconds, after which the cells were immediately placed on ice for 2 minutes. 250µl pre-warmed SOC media was added and this was incubated at 37^{0}C for 1hour 30 min with shaking. After incubation, 100µl of the transformation mix was plated onto Luria-Bertani agar plates which contained 75µg/ml ampicillin.

2.6.3 Cloning into pET 24a Expression Vector

In order to facilitate ligation of insert DNA with plasmid vector DNA, pET® 24a was digested with XhoI and NdeI in a reaction which was set up as follows: plasmid DNA (10µl), XhoI (2µl), NdeI (2µl), reaction buffer 10X (4µl), dH_2O (22µl). Digestion was performed at 37^{0}C for 5hrs.

2.6.3.1 Ligation

A ligation reaction was set up as follows: Ligation Buffer (3µl), dH_2O (4.5µl), plasmid DNA (pET® 24a 12.5µl), insert DNA (8µl), ligase (2µl), and left at room temperature overnight.

2.6.3.2 Transformation of pET® 24a

5µl of the ligation reaction was added to 50µl TOPO® TOP 10 F´ cells (or 50µl DHα5 competent cells). This was placed on ice for 30 minutes. The cells were then heat shocked by placing the tube in a water bath at 42^{0}C for 45 seconds, after which the cells were immediately placed on ice for 2 minutes. 250 µl pre-warmed SOC media was added and this was incubated at 37^{0}C for 1 hour 30 min with shaking. After incubation, 100µl of the transformation mix was plated onto Luria-Bertani agar plates which contained 75µg/ml ampicillin.

2.6.4 Cloning into pCR®T7/NT

The PCR product was cloned directly into the pCR®T7/NT expression vector after purifying it from the agarose gel by ultra-filtration using an Ultrafree-DA kit. The pCR®T7/NT TOPO-TA Cloning kit (Invitrogen) was used and protocols as described in the instuction manual were followed.

Reaction mixture included: PCR product (3µl), salt solution (1µl) and TOPO® vector (pCR®T7/NT, 1µl) The reaction mixture was mixed gently and left at room temperature for 20 minutes.

2.6.4.1 Transformation of pCR®T7/NT

2µl of the plasmid mix was added to 50µl TOPO® cells. This was placed on ice for 30 minutes. The cells were then heat shocked by placing the tube in a water bath at 42^0C for 45 seconds, after which the cells were immediately placed on ice for 2 minutes. 250 µl pre-warmed SOC media was added and this was incubated at 37^0C for 1hour 30 min with shaking. After incubation, 100µl of the transformation mix was plated onto Luria-Bertani agar plates which contained 75µg/ml ampicillin.

2.6.4.2 Colony PCR

Transformed colonies were picked and screened by colony PCR. The reaction mixture contained:

T7 polymerase primer (0.5µl), 3'primer (XhoI) (0.5µl), 10X reaction buffer (5µl), dNTPs (12mM) (1µl), Taq (0.5µl) and dH_2O to a final volume of 50µl.

2.6.4.3 Plasmid Isolation

Plasmid isolation was performed using a Mini-Prep Kit (Sigma) as described in the instruction manual.

2.6.5 Purification of DNA from Agarose

In order to purify the DNA of interest from agarose gels, two methods of purification were employed- one employing the use of silica fines, and the other employing the use of a spin column. In both procedures, the DNA of interest is first excised from the agarose gel.

2.6.5.1 Purification using Silica Fines

NaI solution (1ml) was added to the DNA band of interest. The tube was placed at 65^0C to facilitate melting of the agarose and was occasionally inverted to ensure proper mixing of the NaI solution and the melted agarose. Once the agarose was melted, 20µl silica fines was added to the tube, inverted and left at room temperature for 15 minutes. The mixture of DNA and silica fines was spun down, and the supernatant discarded. The mix of DNA and silica fines was washed twice using ethanol (100%), after which the tubes were left to air-dry for one minute. The silica fines with adsorbed DNA was then resuspended in 20µl dH_2O and the DNA eluted after centrifuging the suspension at maximum speed for 1 minute. The eluted DNA was transferred to a clean eppendorf tube and stored at 4^0C.

2.6.5.2 Purification using a Spin Column

The excised band containing the DNA of interest was placed in the Ultrafree- DA spin column and centrifuged at 10 000rpm (Eppendorf Centrifuge 5415 D) for 10 minutes. Eluted DNA was stored at 4^0C.

2.6.6 Restriction Digestion

Restriction digest of plasmids with enzymes NdeI and XhoI was performed in a reaction with a total volume of 40µl as follows: 10µl plasmid DNA, 2µl of each enzyme, 4µl 10X reaction buffer, and dH_2O to a final volume of 40µl. Digestion was performed at 37^0C for 4 hours.

2.6.7 Electrophoresis

Samples were electrophoresed on a 1% agarose gel at 120V for 1 hour 30 minutes with λEco 471 as standard molecular weight marker. Gels were developed with EtBr.

2.6.8 Transformation of BL21 (DE3) and BL21(DE3) pLysS cells

The protocol used for transformations was as described in the pCR®T7/NT TOPO®-TA Cloning kit (Invitrogen) instruction manual.

2µl of plasmid DNA and 16µl of competent cells were used. This mixture was incubated on ice for 30 minutes, after which the cells were heat shocked at 42^{0}C for 45 seconds. The cells were immediately transferred to ice, and 250µl pre-warmed SOC media was added. The transformation mix was incubated at 37^{0}C with shaking. After 30 minutes, LB plates and 10ml aliquots of LB broth (containing a final concentration of 75µg /ml ampicillin) were each inoculated with 125µl of the transformation mix. Plates and broth were incubated at 37^{0}C overnight.

2.6.9 Induction of Protein Expression

500µl of the overnight culture of BL21 (DE3) and BL21 (DE3) pLysS transformed cells was used to inoculate 10ml LB broth (containing ampicillin to a final concentration of 75µg/ml). This was incubated at 37^{0}C with shaking, and after 2 hours the culture was split into two 5ml cultures. IPTG (to a final concentration of 1mM) was added to one of the 5ml cultures. 500µl of each culture was removed and centrifuged at maximum speed for 30 seconds The supernatant was discarded. The cell pellets were frozen at -20^{0}C. This served as the zero time point samples. The cultures (one induced and one uninduced) were then incubated at 37^{0}C for 4 to 6 hours, with shaking. After 4 hours, 500µl aliquots were removed from each culture, centrifuged at maximum speed for 30 seconds and the pellets stored at -20^{0}C, to monitor protein expression.

2.6.10 SDS-PAGE

Expression of the induced protein was visualised by running samples on a 15% acrylamide gel. Samples were prepared as follows: The cell pellets, stored at -20^{0}C were resuspended in 80µl sample buffer and aliquots of this electrophoresed. 30µl aliquots of samples were boiled for 5 minutes before being electrophoresed on a 15 % acrylamide gel. Bands were visualized with Coomasie Brilliant Blue.

2.7 Antifungal Bioassay

Plants do not contain an immune system and must rely on other mechanisms to protect themselves from fungal infection. A number of antifungal proteins have been isolated from a variety of plant tissues, of which PIs are included. The possible involvement of trypsin inhibitors in plant defense against fungal pathogens has also been implicated by their antifungal activities. It was shown that purified trypsin inhibitor inhibited both *in vitro* conidia germination and hyphal growth of *Aspergillus flavus*. However, the mode of action of these proteins has not been clearly demonstrated (Chen *et al.*, 1999b). PIs from buckwheat were able to suppress germination of *Alternaria alternata* and *Fusarium oxysporum* spores and also inhibit proteases secreted by these fungi. The inhibitors were also capable of inhibiting hyphal elongation of the fungi (Dunaevsky *et al.*, 1998).

Fungal isolates used in the assay included *Fusarium verticilliodes* MRC 826, *Fusarium oxysporum* V-101P- E(1)2, *Fusarium pseudograminearum* MRC 6303, *Botrytis cinerea*, *Eutypa lata* (EL 4) and *Penicillium expansum* which were obtained from the Medical Research Council (MRC) and Agricultural Research Council (ARC) culture collections. Fungi were maintained on Carnation Leaf agar at 4^0C after growth on CLA for 14 days at 27^0C under a 12 hour photoperiod.

2.7.1 Effect of Protease Inhibitors on Germination of Fungal Conidia

Fungal Isolates:

Conidium concentrations of test cultures were adjusted to $1x10^6$ conidia/ml and used in assays. A concentration of $1x10^6$ conidia/ml was obtained by adding 5ml sterile distilled Tween 80 water to the fungal culture on a CLA plate and gently shaking the plate to get the spores into suspension. The suspension was then filtered and the density adjusted microscopically to $1x10^6$ conidia/ml with the aid of a Neubaeur haemocytometer.

Standardization of purified wheat trypsin inhibitor (TI) solutions:

Dilutions of TI at concentrations of 0.25mg/ml, 0.5mg/ml and 1mg/ml were prepared from a stock concentration of 3mg/ml. The solutions were filter sterilized using millipore filters (pore-size 0.22µm) and kept at 4^0C.

Procedure:

For each of the fungal isolates tested, 100µl of the respective standardized conidium suspension was spread onto water agar plates and allowed to dry. Following this, 100µl of the respective TI concentration was spread onto the plate. Three control plates, containing no TI solution were also prepared (for each isolate). Conidia were allowed to germinate at 27^0C for 12 hrs. For each TI treatment the germ tube length of 50 randomly selected germinating conidia was measured using a stereo microscope fitted with an ocular micrometer. The eyepiece was calibrated using a graticule for each objective used.

Chapter 3

Results and Discussion:

Isolation and Purification of Inhibitors

Chapter3
Results and Discussion:
Isolation and Purification of Inhibitors

3.1 Isolation and Purification of Subtilisin Inhibitors

3.1.1 Isolation and Purification Procedures

3.1.1.1 Isolation 1

3.1.1.1.1 Extraction

The extraction procedure as described in section 2.1 was followed, except that 80g wheat seed was used as starting material.

3.1.1.1.2 Subtilisin-Affinity Chromatography

The method employed for subtilisin-affinity chromatography was modified from that described by Mosolov and Shul'gin (1986).

100ml of the dialysate (Fraction 1) obtained after extraction, was mixed with 12ml Subtilisin-Sepharose 4B Affinity Resin, previously equilibrated with 0.1M Sodium phosphate buffer, (pH6.8) (Buffer A). The mixture of dialysate and affinity resin was left rotating end-over-end at 4^0C, overnight. After centrifugation at 10 000 rpm for 5 minutes, the supernatant was collected and stored as the "unbound/ unreactive subtilisin fraction" (Fraction 1-A). The resin was washed with 50ml aliquots of Buffer A containing 1M NaCl until the UV absorbance at 280nm measured approximately 0.06. The resin was then washed with 50ml 8M urea, and the bound PIs eluted batchwise in 12ml 0.2M KCl (pH2) (Fraction 1-H). All fractions were collected and stored at 4^0C. SDS-PAGE analysis was performed as described in section 2.5.1. (Figure 3.1).

3.1.1.2 Isolation 2

3.1.1.2.1 Extraction

The extraction procedure as described in section 2.1 was followed using 500g wheat seeds as starting material.

3.1.1.2.2 Subtilisin- Affinity Chromatography

188ml of the dialysate (Fraction 2) obtained after extraction was applied to 12ml subtilisin Sepharose 4B Affinity Resin, previously equilibrated with Buffer A. The mixture of dialysate and affinity resin was left rotating end-over-end at 4^0C, overnight. After centrifugation at 10 000 rpm for 5 minutes, the supernatant was collected and stored as the "unbound/unreactive fraction" (Fraction 2-A). The resin was washed with 50ml aliquots of Buffer A containing 1M NaCl until the UV absorbance at 280nm measured approximately 0.06. The resin was then washed with 50ml 8M urea, and the bound PIs eluted batchwise in 12ml 0.2M KCl (pH2) (Fraction 2-H). All fractions were stored at 4^0C. Protein concentrations of collected fractions were determined and SDS-PAGE analysis performed (Figure 3.2) as described in sections 2.3.1 and 2.5.1. respectively. The inhibitory activity of collected fractions against commercial trypsin was determined as described in section 2.3.2.

3.1.1.2.3 Cation-Exchange Chromatography

5ml of the 0.2 M KCl (pH 2) eluent (Fraction 2-H) from the subtilisin-affinity resin was applied to a 20 HS POROS column (described in section 2.2.2.1), equilibrated with 0.1M Na acetate buffer (pH 4). The column was developed with a stepwise gradient of 0.1M Tris-HCl buffer (pH8) and 0.1M Tris-HCl buffer (pH8) containing 1M and 2M NaCl, respectively at a flow rate of 10ml/min. Fractions were eluted at a rate of 1ml/min and monitored by UV absorbance at 280nm for protein content (Figure 3.3). Collected fractions (1ml) were stored at 4^0C and protein concentrations of all fractions were determined as described in section 2.3.1. The pooled fractions (2-H-1and 2-H-2) were further purified by HPLC.

3.1.1.2.4 HPLC

The pooled fractions (2-H-1and 2-H-2) from the 20 HS POROS DEAE column were further purified by applying 100µl to a C-18 HPLC column using a solvent gradient as follows: 0% solvent B for 5 minutes, 0- 30% solvent B over 10 minutes, 30-50% solvent B over 30 minutes and 50-100% solvent B over 10 minutes. Eluting peaks (Fractions 2-H-1 and 2-H-2) were collected and used in mass spectroscopy analysis as described in section 2.5.2 (Figures 3.4a and b) A trypsin digest of the HPLC purified inhibitor was also performed, and the resultant peptide profile obtained using mass spectroscopy (Figure 3.5) and analysed using MS-Fit.

Results

SDS-PAGE analysis of the 0.2M KCl eluent (Fraction 1-H) from the subtilisin affinity chromatography revealed the presence of a protein band which corresponded in size (approximately 20 000Da) with previously described PIs isolated from wheat (Mosolov and Shul'gin, 1986). The calculated molecular masses for three bands observed on SDS-PAGE (Figure 3.1) are 21 900, 17 400 and 10 500 Da. The starting material for isolation 1 was only 80g of milled wheat, and since a protein in the expected size range was observed on the gel, it was decided to increase the amount of starting material and attempt to isolate a larger quantity of the PI for kinetic analysis and bioassays to determine the potential as antifungal or insecticidal agents. In isolation 2, subtilisin affinity chromatography was used to separate the PIs from other contaminating proteins, but only 3.6mg of protein without any inhibitory activity against commercial trypsin was detected (Table 3.1).

SDS-PAGE analysis indicated the presence of one protein band with molecular mass of approximately 13 200 Da (Figure 3.2). Cation-exchange chromatography of the affinity-purified inhibitor was performed to attempt the separation of possible isoforms as reported for buckwheat seeds (Dunaevsky *et al.*, 1998). Two peaks were obtained, the first protein peak eluting with the pH gradient (Fraction 2-

H-1), and the second protein peak eluting with the salt gradient (Fraction 2-H-2) (Figure 3.3). Both fractions were subjected to HPLC and analysed by MALDI TOF, which revealed the presence of proteins with molecular masses of 13 282 and 12 081 Da, respectively (Figure 3.4 a and b). Analysis of the protein with molecular mass of 13 282 Da (Fraction 2-H-1) by trypsin digest, Mass spec analysis (Figure 3.5) and subsequent homology sequence searches using the MS-Fit programme, indicated that the protein was homologous to a wheat α-amylase/trypsin inhibitor (SwissProt accession number P16159). Fraction 2-H-2 was not subjected to trypsin digestion due to insufficient amounts of protein in the sample.

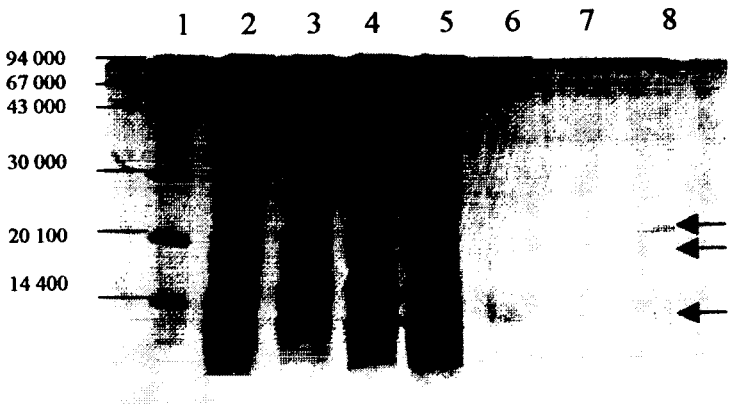


Figure 3.1 SDS-PAGE of fractions from the initial purification procedure using 80g wheat seed as starting material. Lane 1: Molecular weight marker; Lane 2: Crude wheat extract: Lane 3:Overflow of lane 2; Lane 4: Dialysed wheat extract (Fraction 1); Lane 5:Fraction 1-A; Lane 6: NaCl wash; Lane 7: 8M Urea wash; Lane 8: KCl eluent (Fraction1-H). 10µl of each sample was loaded onto the 4% stacking, 15% resolving gel. Gels were electrophoresed at 200 V for 45 min, and proteins visualised with Coomassie Blue. Arrows indicate 3 bands with calculated M_r values of 21 900, 17 400 and 10 500, respectively.

Table 3.1 Purification of Subtilisin Inhibitors from *Triticum aestivum* using 500g wheat seed as starting material. Wheat seeds were extracted in 0.1M Sodium phosphate buffer (pH6.8)

Sample	Tot.Vol (ml)	Protein (mg/ml)	Tot. Protein (mg)	Activity (ΔA410nm/ min/0.1ml)	Tot. Activity (Inhib.Units)	Spec. Activity (Units/mg protein)	Purification Factor	Yield (%)
Fraction-2	188	24	4512	3.1	5828	1.29	1	100
Fraction 2-A	165	23	3795	2.84	4686	1.23	0.96	80
Fraction 2-H	12	0.3	3.6	NAD	-	-	-	-
Fraction 2-H-1	0.5	0.02	0.01	NAD	-	-	-	-
Fraction 2-H-2	0.5	0.03	0.015	NAD	-	-	-	-

NAD: no activity detected

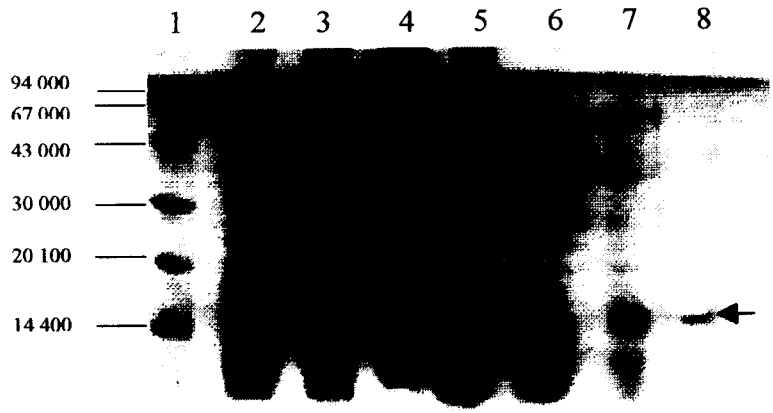


Figure 3.2 SDS-PAGE of Fractions from Isolation 2. Lane 1: Molecular weight markers; Lane 2: crude wheat extract, Lane 3: dialysed wheat extract (Fraction 2); Lane 4: Dialysed wheat extract (Fraction 2); Lane 5: Fraction 2-A; Lane 6: NaCl wash; Lane 7: 8M urea wash. Lane 8: 0.2M KCl eluent (Fraction 2-H). 10µl of each sample was loaded onto the 4% stacking, 15% resolving gel. Gels were electrophoresed at 200 V for 45 min, and proteins visualised with Coomassie Blue.

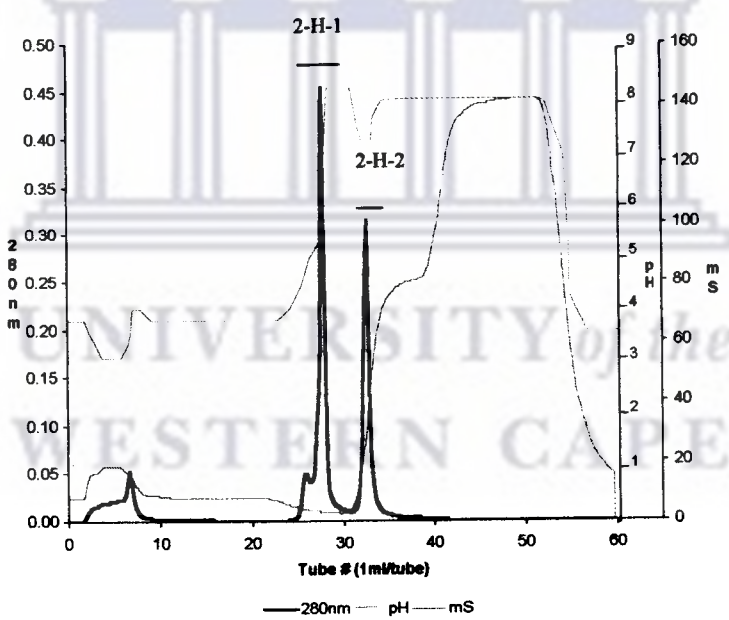


Figure 3.3 Cation exchange chromatography of Fraction 2-H (5ml). The column was equilibrated with 0.1M Sodium Acetate buffer (pH 4) and developed with a stepwise gradient of 0.1M Tris-HCl buffer (pH 8) and 0.1M Tris-HCl buffer (pH 8) containing 1 M and 2M NaCl, respectively. Fractions were pooled as indicated (2-H-1 and 2-H-2). Black line indicates A_{280nm} profile; Red line indicates pH gradient; Blue line indicates salt gradient.

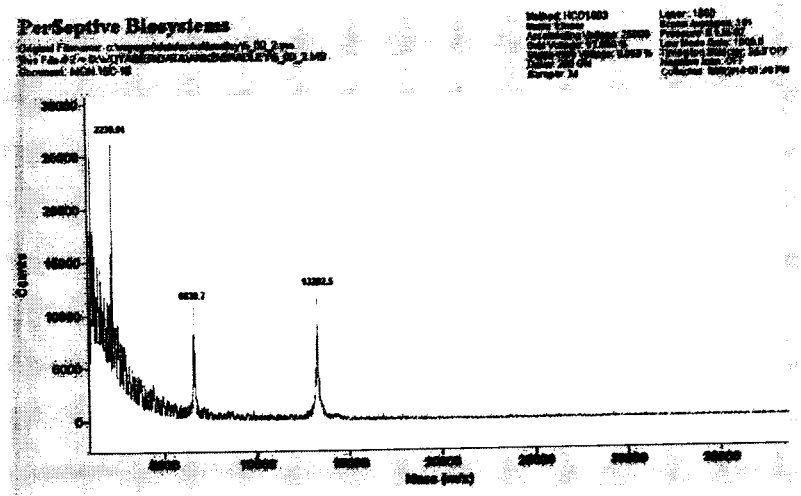


Figure 3.4(a) MALDI TOF mass spectrum of Fraction 2-H-1 obtained from cation exchange chromatography of Fraction 2-H.

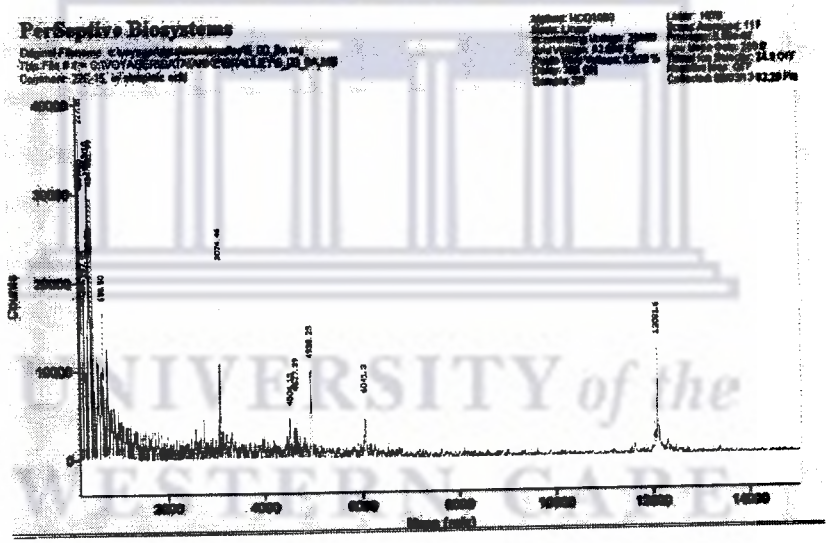


Figure 3.4 (b) MALDI TOF mass spectrum of Fraction 2-H-2 obtained from cation exchange chromatography of Fraction 2-H.

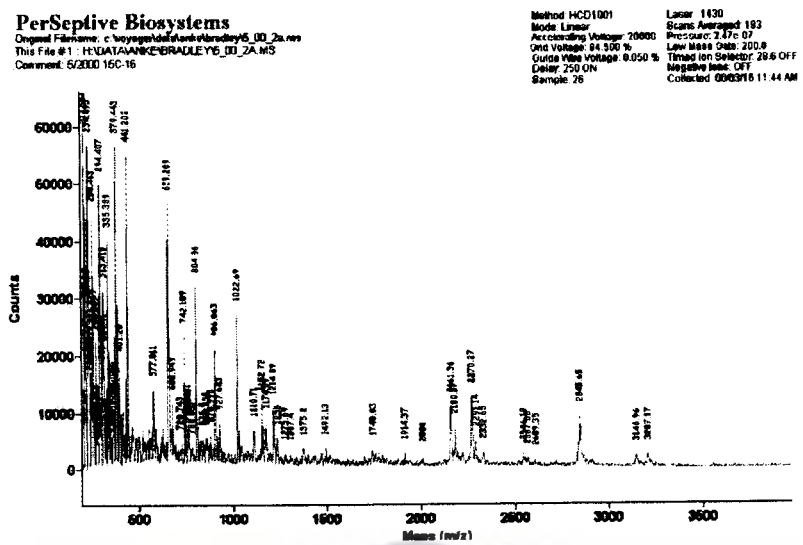


Figure 3.5 MALDI TOF mass spectrum of trypsin digest of Fraction 2-H-1

Discussion

Mosolov and Shul'gin (1986) isolated specific subtilisin inhibitors from rye wheat and triticale using subtilisin affinity chromatography. The subtilisin inhibitors had molecular mass values of approximately 20 000 Da and were very strong inhibitors of subtilisins Carlsberg and BPN', while exhibiting no inhibitory activity against trypsin, chymotrypsin or pancreatic elastase. Previous to this Mundy *et al.* (1984) had isolated the first bifunctional α-amylase/subtilisin inhibitor from wheat. This protein had a molecular mass of approximately 20 500 Da and inhibited germination specific α-amylases of cereals. They also isolated proteins which inhibited animal α-amylases specifically, and which had molecular mass values of approximately 13 000–15 000 Da. Walsh and Twitchell (1991) reported the purification of a 20 500 Da PI from potato tubers which inhibited the microbial protease subtilisin Carlsberg. This PI was shown to have significant homology to other Kunitz inhibitors. The molecular masses of 13 282 Da (Fraction 2-H-1) and 12 081 Da (Fraction 2-H-2) would suggest that these wheat protease inhibitors belong to the α-amylase specific inhibitors, whereas MS Fit analysis of the mass spec profile of the trypsin digest of fraction 2-H-1 would suggest that it is homologous to the

wheat α-amylase/trypsin inhibitor P16159. Both fractions (2-H-1 and 2-H-2) bound to the subtilisin–affinity resin, which would indicate that they inhibit subtilisin. Activity assays against trypsin indicate that the proteins did not inhibit trypsin.

The SDS-PAGE analysis of isolation 2 failed to show a band with M_r of 20 000, as was observed with isolation 1. Instead, the KCl eluent displayed a band with M_r of 14 000, which was observed in SDS analysis of Isolation 1, but was not as intense as in Isolation 2. However, both isolations yielded proteins, which fall in the molecular mass range of known wheat protease inhibitors. The low yield of inhibitors from the subtilisin affinity chromatography could be due to the low prevalence of inhibitors in wheat or to denaturation under the elution conditions, which also results in irreversible activity loss of the immobilized subtilisin. The majority of trypsin inhibitory activity (4686 units) did not bind to the subtilisin affinity column. An alternative affinity chromatography was therefore attempted.

3.2 Isolation and Purification of Trypsin Inhibitors

3.2.1 Isolation and Purification Procedures

3.2.1.1 Isolation 1

Dunaevsky *et al.* (1998) reported the isolation of protease inhibitors from buckwheat seeds using a Trypsin Sepharose column. It was therefore decided to use a trypsin affinity column for the next step in the isolation of inhibitors from the wheat extract. The method employed was modified from that described by Dunaevsky *et al.* (1998), using a Bio-RAD Econo System with a Model 2110 Fraction collector.

3.2.1.1.1 Trypsin Affinity Chromatography

Fraction 2-A (165ml), the unbound fraction from subtilisin affinity resin, was applied to a trypsin Sepharose 4B affinity column previously equilibrated with buffer A. Equilibration was achieved by washing the column with 5 column volumes of buffer A at a flow rate of 1ml/min. The sample was applied to the column at a rate of 1ml/min. The column was developed with 5 column volumes of buffer A, and then with 5 column volumes of buffer A containing 0.5M NaCl to remove unbound proteins. The eluent was monitored by UV absorbance at 280nm for protein content. PIs were eluted from the immobilized trypsin with 150ml 1mM HCl containing 0.5M NaCl at a flow rate of 1ml/min (Figure 3.6). Protein concentrations of collected fractions were determined and SDS-PAGE analysis performed (Figure 3.7a and b) as described in sections 2.3.1 and 2.5.1, respectively. The inhibitory activity of collected fractions against commercial trypsin (using BAPNA as substrate) was determined as described in section 2.3.2. Eluted fractions were then pooled according to protein and trypsin inhibitory activity profiles (Fractions 2-A-1, 2-A-2 and 2-A-3, Figure 3.6) and subjected to ion exchange chromatography (Figure 3.8). Fraction 2-A-2 was also analysed by mass spectroscopy on a MALDI TOF (Figure 3.9a).

3.2.1.1.2 Cation Exchange Chromatography

Cation exchange chromatography was performed on 1ml of Fraction 2-A-2 using a 20 HS POROS column (described in section 2.2.2.1), equilibrated with 0.1M Sodium actetate buffer (pH4). The column was developed with a stepwise gradient of 0.1M Tris-HCl buffer (pH8) and 0.1M Tris-HCl buffer (pH8) containing 1M and 2M NaCl, respectively at a flow rate of 10ml/min. The eluent was monitored at 280nm for protein content. No protein bound to this column.

3.2.1.1.3 Anion Exchange Chromatography

Anion exchange chromatography was performed on 1ml of Fraction 2-A-2 using a 20 HQ POROS column as described in section 2.2.2.2. Fractions were eluted at a rate of 1ml/min and pooled according to protein profiles (Fractions 2-A-2-A, 2-A-2-B and 2-A-2-C, Figure 3.8). Mass spec analysis was performed using fraction 2-A-2-B as described in section 2.5.2.1 (Figure 3.9b). CNBr cleavage was performed on Fraction 2-A-2-B. The resultant peptide profile, obtained using mass spectroscopy (Figure 3.10) was analysed using MS Fit.

3.2.1.2 Isolation 2

To obtain sufficient quantity of trypsin inhibitor for bioassays, a second preparative isolation was attempted, eliminating the subtilisin affinity chromatography step.

3.2.1.2.1 Extraction

The extraction procedure as described in Section 2.1 was followed, using 500g wheat seed as starting material. The seed was extracted with 0.1M Sodium phosphate buffer (pH6.8) (buffer A) and the clarified extract dialysed against buffer A at $4^{O}C$ until the dialysate reached pH6.8 (Fraction 3).

3.2.1.2.2 Trypsin Affinity Chromatography

Fraction 3 (250ml) was applied to the trypsin affinity column (2.6x4.3cm), previously equilibrated with buffer A, at a flow rate of 1ml/min. The column was developed with 5 column volumes of buffer A, and then with 5 column volumes of buffer A containing 0.5M NaCl to remove unbound proteins. The eluent was monitored by UV absorbance at 280nm for protein content. PIs were eluted from the immobilized trypsin with 150ml 1mM HCl containing 0.5M NaCl at a rate of 1ml/min. Fractions (1ml) were collected. The Bradford assay and trypsin inhibitory activity assays (using BAPNA as substrate) were performed on eluted fractions as described in sections 2.3.1 and 2.3.2, respectively. Eluted fractions were then pooled according to protein and trypsin activity profiles (Fractions 3-A, 3-B and 3-C Figure 3.11) and analysed by SDS-PAGE (Figure 3.12) as described in Section 2.4.1. Anion exchange chromatography was performed (using 1ml of Fraction 3-B) as described in section 2.2.2.2. (Figure 3.13). Fraction 3-B was dialysed against distilled water at $4^{O}C$ for 5hrs and then lyophilized. An aliquot of dialysed Fraction 3-B was analysed by mass spectroscopy (Figure 3.14). Fraction 3-B was stored at $-20^{O}C$ in the lyophilized form until analysed for inhibitory activity against insect gut protease and antifungal activity.

3.2.1.3 Isolation 3

To obtain sufficient quantity of trypsin inhibitor for kinetic characterization and bioassays, a third preparative isolation was performed as described in Isolation 2, Section 3.2.1.2.

3.2.1.3.1 Extraction

The extraction procedure as described in Section 2.1 was followed, using 500g wheat seed as starting material. The seed was extracted with 0.1M Sodium phosphate buffer (pH6.8) (buffer A) and the clarified extract dialysed against buffer A at 4^{O}C until the dialysate reached pH6.8 (Fraction 4).

3.2.1.3.2 Trypsin Affinity Chromatography

Fraction 4 (237ml) was applied to the trypsin affinity column (2.6x4.3cm), previously equilibrated with buffer A, at a flow rate of 1ml/min. The column was developed with 5 column volumes of buffer A, and then with 5 column volumes of buffer A containing 0.5M NaCl to remove unbound proteins. The eluent was monitored by UV absorbance at 280nm for protein content. PIs were eluted from the immobilized trypsin with 150ml 1mM HCl containing 0.5M NaCl at a flow rate of 1ml/min. Fractions (1ml) were collected. The Bradford assay, trypsin inhibitory activity assays (using both BAPNA and TAME as substrate) and chymotrypsin inhibitory activity assays (using ATEE as substrate) were performed on eluted fractions as described in sections 2.3.1 and 2.3.2. respectively. Eluted fractions were then pooled according to protein and trypsin activity profiles (Fractions 4-A, 4-B and 4-C, Figure 3.15) and analysed by SDS-PAGE (Figure 3.12) as described in Section 2.5.1. Fraction 4-B was dialysed against distilled water, at 4^{O}C for 5hrs and an aliquot subjected to mass spectroscopy analysis (Figure 3.16). Fraction 4-B was lyophilized after dialysis and stored at -20^{O}C in the lyophilized form until used for kinetic characterization and antifungal bioassays.

Results

The trypsin affinity column elution profile of Fraction 2-A shows one major protein peak (Figure 3.6) which corresponded with eluted fractions that show a decrease in trypsin activity (i.e. an increase in trypsin inhibitory activity). Eluted fractions were pooled as indicated according to their protein and trypsin inhibitory activity profiles. The overall recovery of trypsin inhibitory activity for Fractions 2-A-1, and 2-A-2 was 1.3% and 3.6%, respectively, while the specific activity of these fractions was 24 times and 19 times higher, respectively, when compared to that of the crude wheat extract (Table 3.2). SDS PAGE analysis of the fractions showed the presence of at least six protein bands with M_r of 12 800, 10 800, 9 600, 8 800, 8 100 and 7 200 (Figure 3.7a). These proteins could not be separated by either anion or cation exchange chromatography, with proteins eluting as one major peak when subjected to anion exchange chromatography (Figure 3.8). SDS-PAGE of pooled fraction 2-A-2 revealed a protein with M_r of approximately 6 500 (Figure 3.7b). Initial MALDI TOF analysis of Fraction 2-A-2 showed the presence of a protein with M_r of 7 806 (Figure 3.9.a) which is in contrast to the six bands observed on SDS PAGE. Subsequent mass spec analysis of Fraction 2-A-2-B revealed the presence of a single protein with molecular mass of 7 810 (Figure 3.9b). Mass spectroscopy of the resulting peptide fragments obtained from CNBr cleavage of this protein (Figure 3.10) and subsequent homology searches using MS-Fit, indicated that this protein was homologous to a subtilisin/chymotrypsin inhibitor.

The trypsin affinity column elution profile of Fraction 3, as with the previous isolation of trypsin inhibitors, showed one major protein peak (Figure 3.11), which corresponded with eluted fractions that show an increase in trypsin inhibitory activity. Eluted fractions were pooled as indicated according to protein and trypsin inhibitory activity profiles (Figure 3.11) The overall recovery of trypsin inhibitory activity for Fraction 3-B was 5.4% with specific activity approximately 10 times higher than that of the crude extract (Table 3.3). SDS-PAGE analysis of Fraction 3-B revealed the presence of at least four protein bands with M_r values of 17 000, 13 800, 10 200 and 8 000 (Figure 3.12). When 100µl of

Fraction 3-B was applied to an anion exchange column, proteins were eluted as three peaks (Fractions 3-B-1, 3-B-2 and 3-B-3, Figure 3.13), corresponding to Fractions 2-A-2-A, 2-A-2-B and 2-A-2-C, respectively of Isolation 2. Peaks 3-B-1 and 3-B-3 were considered to be artifacts, as in isolation 2 (Figure 3.8) and were only significant here due to the small amount of protein loaded onto the column. Mass spec analysis of Fraction 3-B (Figure 3.14) revealed the presence of a single protein of molecular mass in the range of 7 809 Da and two minor proteins of molecular mass 13 326 Da and 15 811 Da, respectively, similar to that obtained in isolation 2 (Figure 3.9). The trypsin affinity column elution profile of Fraction 4 (Section 3.2.1.3) showed one major protein peak (Figure 3.15), which corresponded with eluted fractions that show an increase in trypsin inhibitory activity. Eluted fractions were pooled as indicated according to protein and trypsin inhibitory activity profiles. A 15.6 times purification was achieved with a specific activity of 25 Units/mg protein and a yield of 13% (Table 3.4). No chymotrypsin inhibitory activity was observed. SDS-PAGE of Fraction 4-B revealed the presence of 4 protein bands with M_r of 17 000, 13 800, 10 200 and 8 000 (Figure 3.12). Mass spec analysis of Fraction 4-B (Figure 3.16) revealed the presence of proteins with molecular mass values of 7 961, 13 319 and 15 516 Da and a spectrum similar to that obtained in isolations 2 and 3 (Figures 3.9 and 3.14).

Table 3.2 Purification of Trypsin Inhibitor (Isolation 1) from *Triticum aestivum* using 500g wheat seed as starting material Wheat seeds were extracted in 0.1M Sodium phosphate buffer (pH6.8).

Sample	Tot. Vol (ml)	Protein (mg/ml)	Tot. Protein (mg)	Activity (A410nm/ min/0.1ml)	Tot. Activity (Inhib.Units)	Spec. Activity (Units/mg protein)	Purification Factor	Yield (%)
Fraction-2	188	24	4512	3.1	5828	1.29	1	100
Fraction 2-A	165	23	3795	2.84	4686	1.23	0.96	80
Fraction 2-H	12	0.3	3.6					
Fraction 2-A-1	16.5	0.15	2.5	0.46	75.9	30.36	23.5	1.3
Fraction 2-A-2	37.5	0.23	8.6	0.56	210	24.4	18.91	3.6
Fraction 2-A-2-B	0.5	0.58	0.29	0.23	1.15	3.9	3.0	0.02

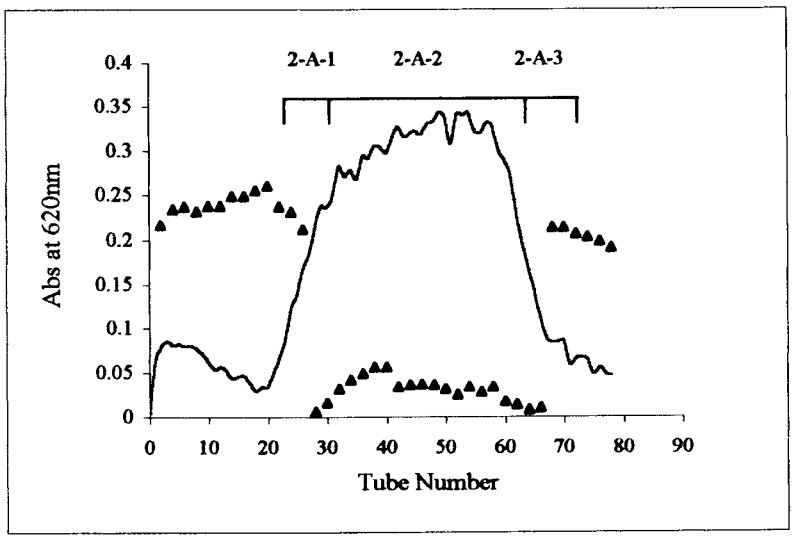


Figure 3.6 Trypsin affinity column elution profile (with 1mM HCl containing 0.5M NaCl) and trypsin inhibitory activity profile of Fraction 2-A. The column (2.6x4.3cm), was equilibrated with 0.1M Phosphate buffer (pH6.8), washed with 0.1M Phosphate buffer (pH6.8) containing 0.5M NaCl and trypsin inhibitory activity eluted with 1mM HCl containing 0.5M NaCl. Protein peaks were detected using the Bradford assay (Blue line). Inhibitory activity was detected by measuring the change in A410nm when using BAPNA as substrate (▲). Fractions 2-A-1, 2-A-2 and 2-A-3 were pooled as indicated.

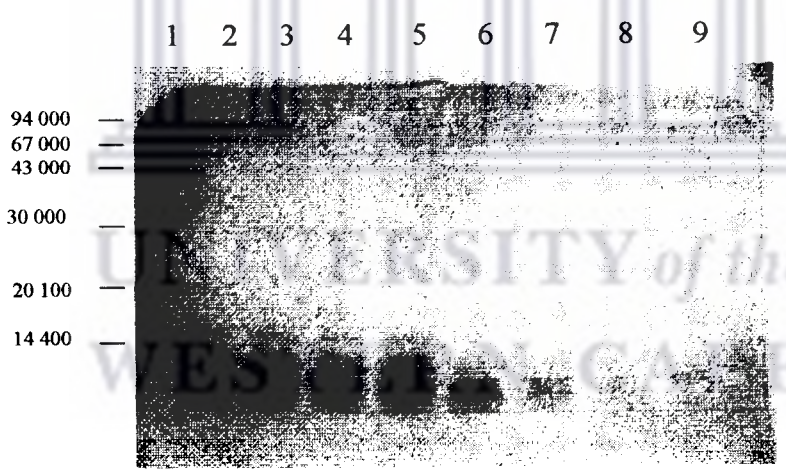


Figure 3.7(a) SDS-PAGE of fractions from trypsin affinity column of Fraction 2-A. Lane 1: Molecular weight marker; Lanes 2-9: tubes (48, 51, 54, 57, 60, 63, 66 and 69) from trypsin affinity column. 10µl of each sample was loaded onto 4% stacking, 15% resolving gel. Gels were electrophoresed at 200 V for 45 min, and proteins visualised with Coomassie Blue.

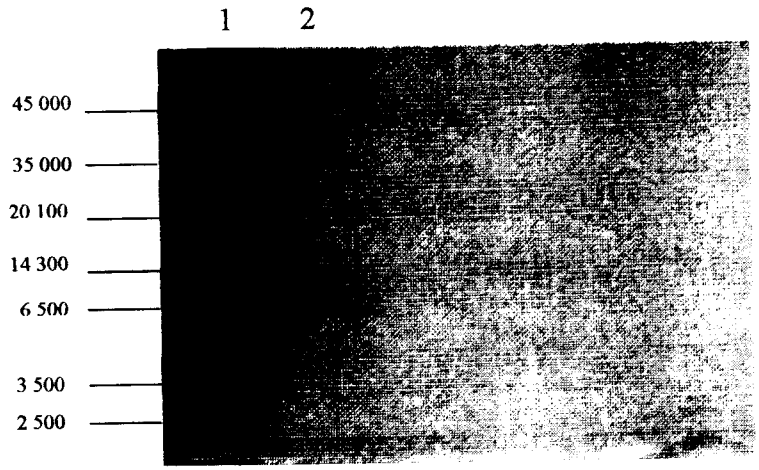


Figure 3.7(b) SDS-PAGE of pooled fraction 2-A-2. Lane 1: Molecular weight markers; Lane 2: Fraction 2-A-2. 10µl of the sample was loaded onto 4% stacking, 15% resolving gel. Gels were electrophoresed at 200 V for 45 min, and proteins visualised with Coomassie Blue.

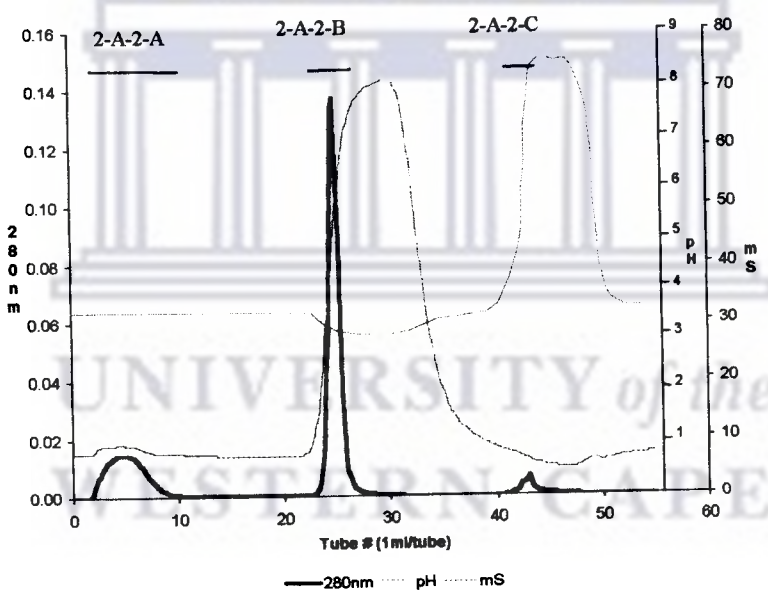


Figure 3.8 Anion exchange chromatography elution profile of Fraction 2-A-2 (1ml). The column was equilibrated with 0.1M Citrate acetate buffer (pH 4) and developed with a stepwise gradient of 0.1M Citrate acetate buffer (pH 4) containing 2M NaCl and 0.1M Tris-HCl buffer (pH8), respectively. Fractions were pooled as indicated (2-A-2-A, 2-A-2-B and 2-A-2-C).

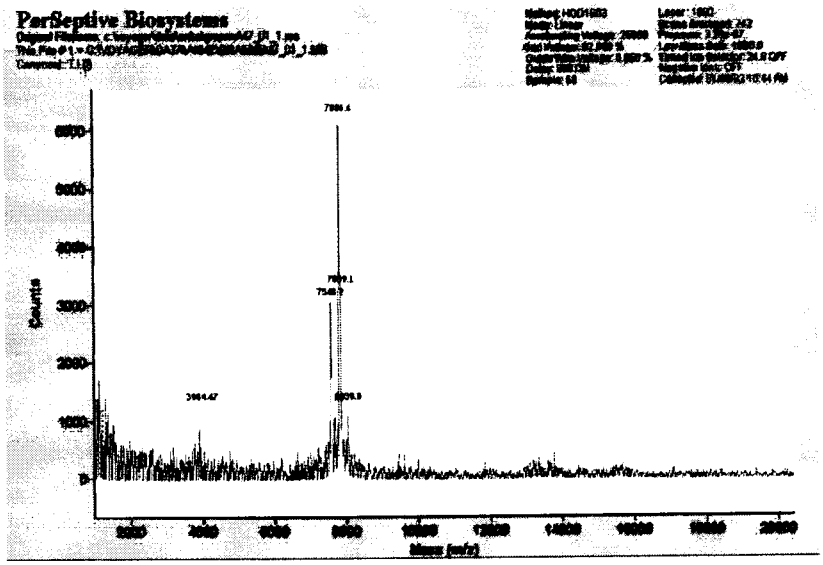


Figure 3.9 (a) MALDI TOF mass spectrum of Fraction 2-A-2 obtained from Trypsin affinity column of Fraction 2-A.

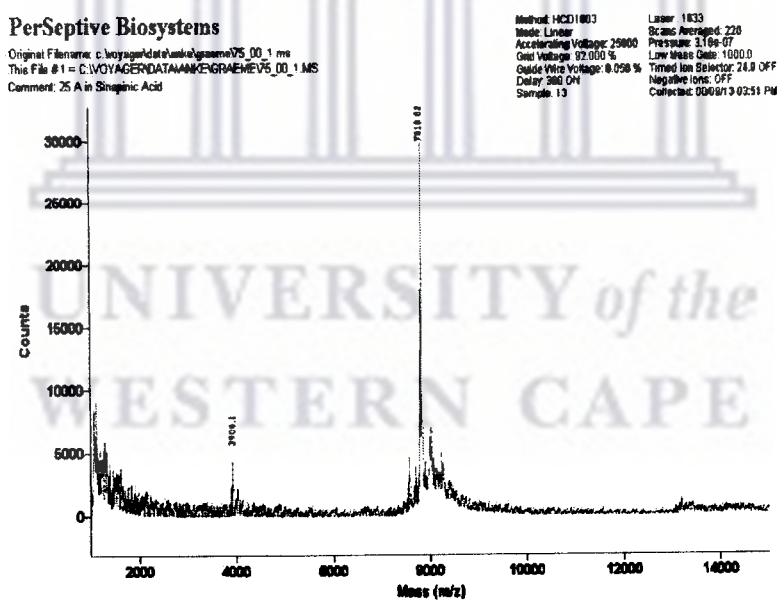


Figure 3.9 (b) MALDI TOF mass spectrum of Fraction 2-A-2-B obtained from anion exchange chromatography of Fraction 2-A-2.

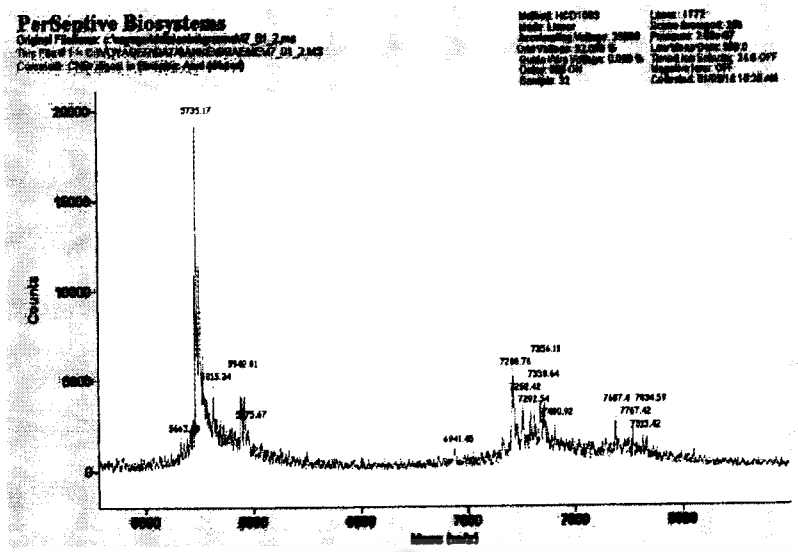


Figure 3.10 MALDI TOF mass spectrum (5000-9000 mass range) of CNBr cleavage of Fraction 2-A-2-B obtained from anion exchange chromatography of Fraction 2-A-2.

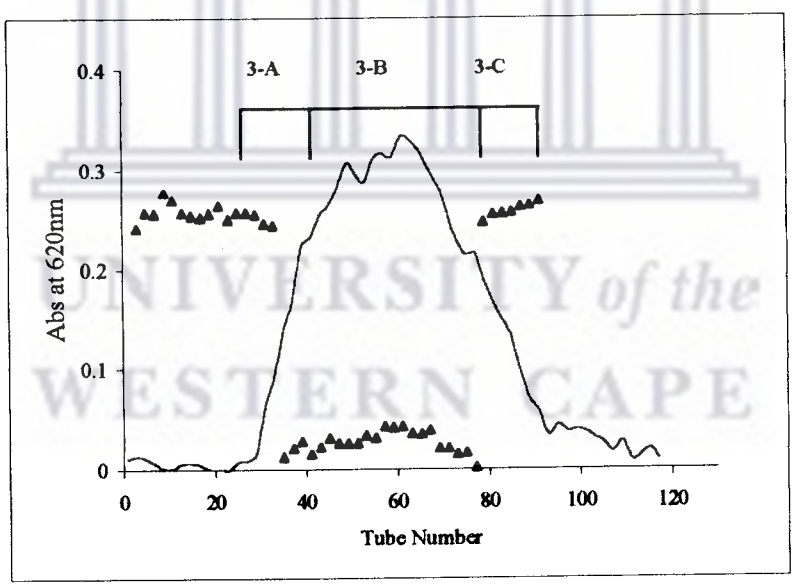


Figure 3.11 Trypsin affinity column elution profile (with 1mM HCl containing 0.5M NaCl) and trypsin inhibitory activity profile of Fraction 3. The column (2.6x4.3cm) was equilibrated with 0.1M Phosphate buffer, (pH6.8), washed with 0.1M Phosphate buffer (pH6.8) containing 0.5M NaCl and trypsin inhibitory activity eluted with 1mM HCl containing 0.5M NaCl. Protein peaks were detected using the Bradford assay (Blue line). Inhibitory activity was detected by measuring the change in A410nm when using BAPNA as substrate (▲). Fractions 3-A,
3-B and 3-C were pooled as indicated.

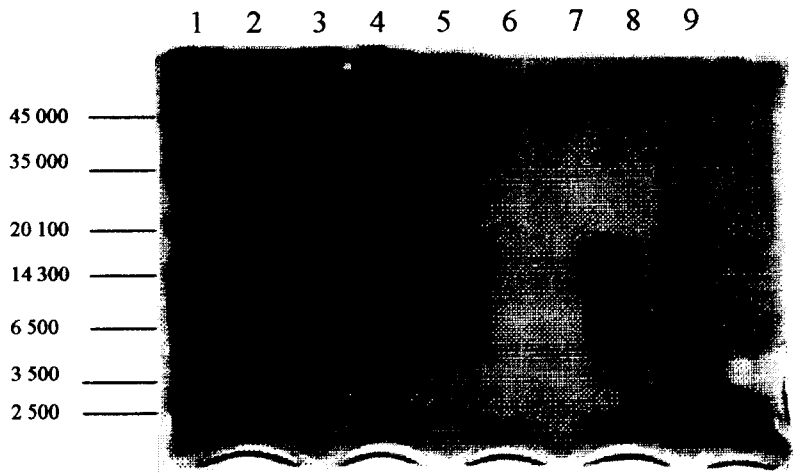


Figure 3.12 SDS-PAGE of Fractions 3-B and 4-B from trypsin affinity columns. Lane 1: molecular weight markers; Lane 2: Crude wheat extract; Lane 3: dialysed wheat extract (Fraction 3); Lane 4: fall through fraction; Lane 5: Fraction 3-B; Lanes 6 and 7- open; Lane 8: Fraction 4-B; Lane 9: Fraction 3-B. 10µl of each sample was loaded onto 4% stacking, 15% resolving gel. Gels were electrophoresed at 200 V for 45 min, and proteins visualised with Coomassie Blue.

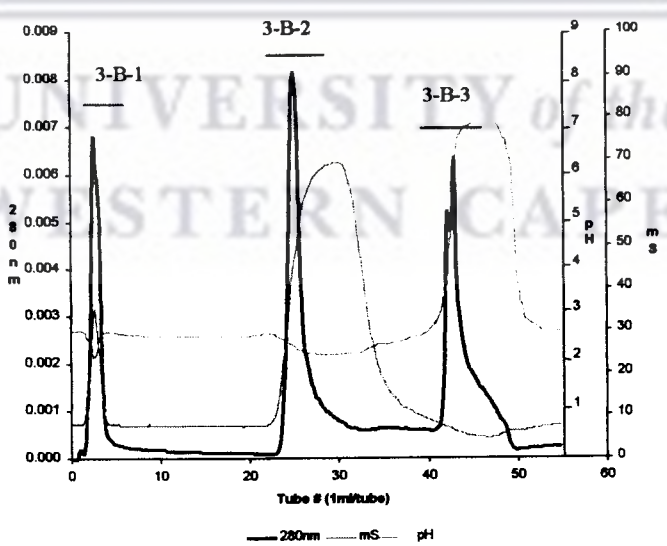


Figure 3.13 Anion exchange chromatography elution profile of Fraction 3-B (100µl). The column was equilibrated with 0.1M citrate acetate buffer (pH 4) and developed with a stepwise gradient of 0.1M citrate acetate buffer (pH 4) containing 2M NaCl and 0.1M Tris-HCl buffer (pH8), respectively. Fractions were pooled as indicated (3-B-1, 3-B-2 and 3-B-3)

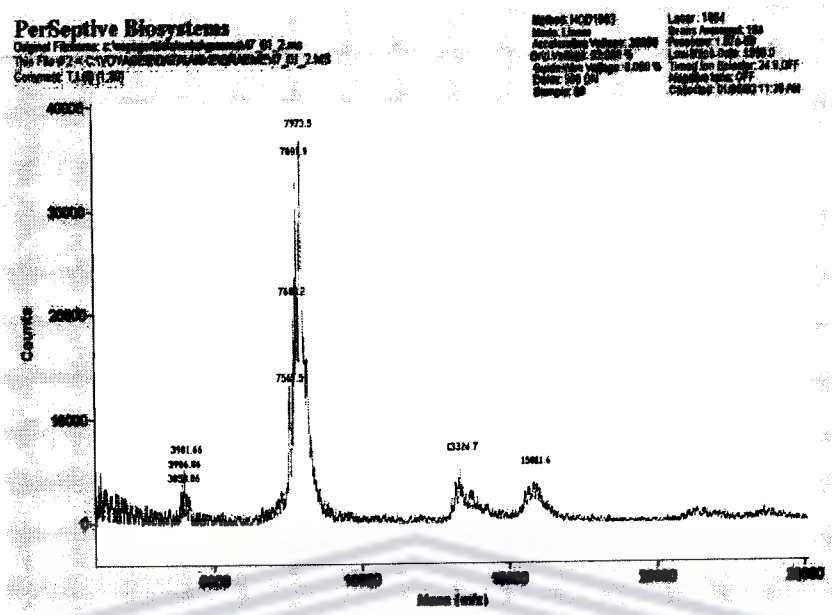


Figure 3.14 MALDI TOF mass spectrum of Fraction 3-B (after dialysis) obtained from trypsin affinity chromatography.

Table 3.3 Purification of Trypsin Inhibitors (Isolation 2) from *Triticum aestivum* using 500g wheat seed as starting material. Wheat seeds were extracted in 0.1M Sodium phosphate buffer (pH6.8)

Sample	Tot.Vol (ml)	Protein (mg/ml)	Tot. Protein (mg)	Activity (ΔA410nm/ min/0.1ml)	Tot. Activity (Inhib.Units)	Spec. Activity (Units/mg protein)	Purification Factor	Yield (%)
Fraction 3	250	6	1500	2.94	7350	4.9	1	100
Unbound	230	5	1150	0.96	2206	1.9	0.39	30
Fraction 3-B	57	0.12	6.84	0.7	399	58.3	11.9	5.4

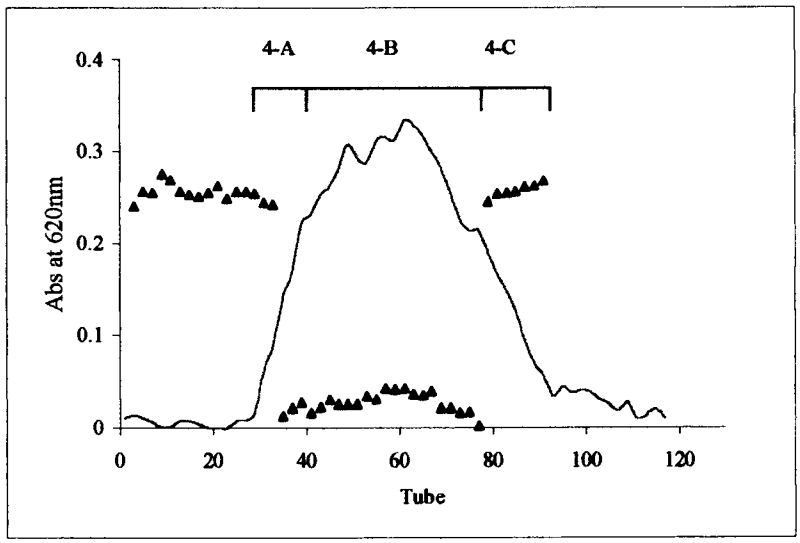


Figure 3.15 Trypsin affinity column elution profile (with 1mM HCl containing 0.5M NaCl) and trypsin inhibitory activity profile of Fraction 4. The column (2.6x4.3cm), was equilibrated with 0.1M Phosphate buffer (pH6.8), washed with 0.1M Phosphate buffer (pH6.8) containing 0.5M NaCl and trypsin inhibitory activity eluted with 1mM HCl containing 0.5M NaCl. Protein peaks were detected using the Bradford assay (Blue line). Inhibitory activity was detected by measuring the change in A410nm using BAPNA as substrate (▲). Fractions 4-A, 4-B and 4-C were pooled as indicated.

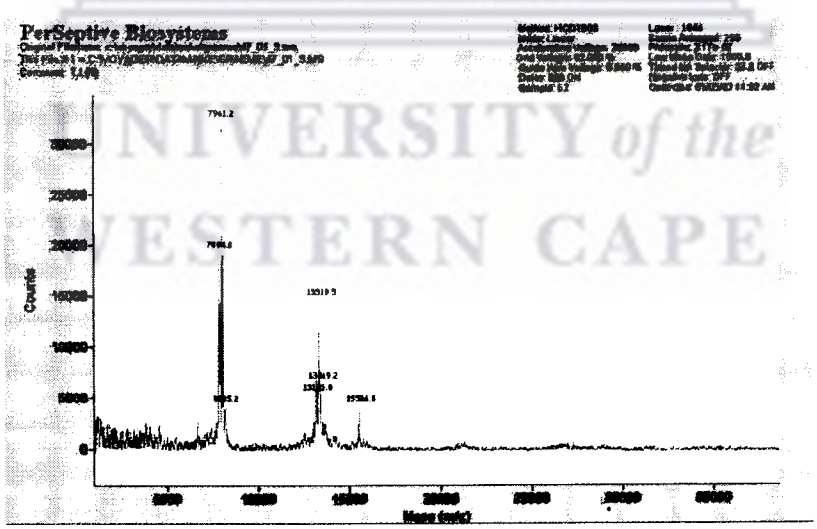


Figure 3.16 MALDI TOF mass spectrum of Fraction 4-B obtained from trypsin affinity chromatography of Fraction 4.

Table 3.4 Purification of Trypsin Inhibitor (Isolation 3) from *Triticum aestivum* using 500g wheat seed as starting material. Wheat seeds were extracted in 0.1M Sodium phosphate buffer (pH6.8)

Sample	Tot. Vol (ml)	Protein (mg/ml)	Tot. Protein (mg)	Activity (ΔA410nm/min/ 0.1ml)	Tot. Activity (Inhib.Units)	Spec. Activity (Units/mg protein)	Purification Factor	Yield (%)
Fraction 4	237	7.63	1808.31	1.2	2844	1.6	1	100
Unbound	217	7.33	1590.61	0.2	434	0.27	0.16	15
Fraction 4-B	48	0.32	15.36	0.8	384	25	15.6	13

Discussion

Standard procedures (i.e. ammonium sulphate precipitation of aqueous extract, followed by affinity and ion-exchange chromatography) were used to extract and purify trypsin inhibitors. The isolation of protease inhibitors was reproducible, with mass spec profiles indicating the same protein (molecular mass 7 807.8 +/-2.2 Da) being present in all three fractions: 2-A-2, 3-B and 4-B. The inhibitor was relatively small (molecular mass approximately 7 800 Da) and inhibited trypsin but showed no inhibitory activity against chymotrypsin. Barber *et al.* (1986) reported the isolation of chloroform methanol (CM) soluble proteins from wheat, with molecular mass values ranging from 11-12 kDa. These CM proteins belong to the α-amylase/ trypsin inhibitor family. Odani *et al.* (1986) also isolated a number of trypsin inhibitors from wheat germ and classified these into 2 groups based on molecular sizes: Inhibitor I (with molecular mass=14 500 Da) and Inhibitor II (with molecular mass =7 000 Da). These inhibitors were shown to be highly homologous to the double-headed protease inhibitors (Bowman-Birk Inhibitors) of Leguminosae plants, although Inhibitors II are single-headed inhibitors which correspond to one inhibitory domain of the Bowman-Birk type double- headed inhibitors. The low molecular weight inhibitor isolated during this study could possibly be placed in the Bowman-Birk type family of inhibitors along with anionic protease inhibitors isolated from buckwheat (Dunaevsky *et*

al., 1998). Dunaevsky *et al.* (1998) placed these inhibitors (which had molecular mass values ranging from 7.7-9.2 kDa and were shown to inhibit trypsin and to a lesser degree chymotrypsin) in the Potato Protease Inhibitor I Family based on amino acid sequence homology. Dunaevsky *et al.* (1995) also reported the isolation of trypsin inhibitors from buckwheat with molecular mass values of 8 700 Da, 9 000 Da and 9 200 Da, while Chen *et al.* (1999b) reported the isolation of a trypsin inhibitor with a molecular mass of 14 kDa from corn.

For both Fractions 2-A-2 and 4-B, the specific activity recovered was similar, although the yield obtained differed vastly, with a 3.6% yield being obtained for fraction 2-A-2, while 13% of the inhibitory activity was recovered from Fraction 4-B. The low yield of inhibitory activity recovered from Fractions 2-A-2 and 3-B compared to that of Fraction 4-B could be due to enzymatic modification of inhibitors during purification procedures. Fraction 2-A-2 was recovered after Fraction 2 (which had not bound to a subtilisin affinity column) was applied to a trypsin affinity column. The possibility exists that prolonged exposure of Fraction 2-A to endogenous proteases, before being applied to the trypsin affinity column, could have resulted in modifications of the inhibitors by these proteases in the sample. It has been a concern that the trypsin inhibitors purified by immobilized trypsin might be proteolytically modified at the reactive sites (Wu and Whitaker, 1990). This could possibly account for the low recovery of trypsin inhibitory activity from Fraction 3-B if the trypsin bound affinity beads were not saturated with inhibitors or if the inhibitors were not eluted from the beads quickly enough. The recovery of inhibitors from different plant sources shows a variation. Broadway (1993) recovered 14% of the trypsin activity from affinity purified cabbage foliage (starting material 500g) and achieved a 67 times purification. The inhibitors were eluted from a trypsin bound affinity column in 8M Urea, pH3. The isolation of anionic inhibitors from buckwheat seeds (starting material 1kg) resulted in an approximate 40 times purification, with yields ranging from 0.6 to 2.6%. Cationic inhibitors from buckwheat were recovered with a 56 times purification and 0.8% yield of inhibitory activity

(Dunaevsky *et al.*, 1998). The yield of four Bowman Birk type protease inhibitors from apple-leaf seed ranged from 9.1-25.7% (Joubert,. 1984).

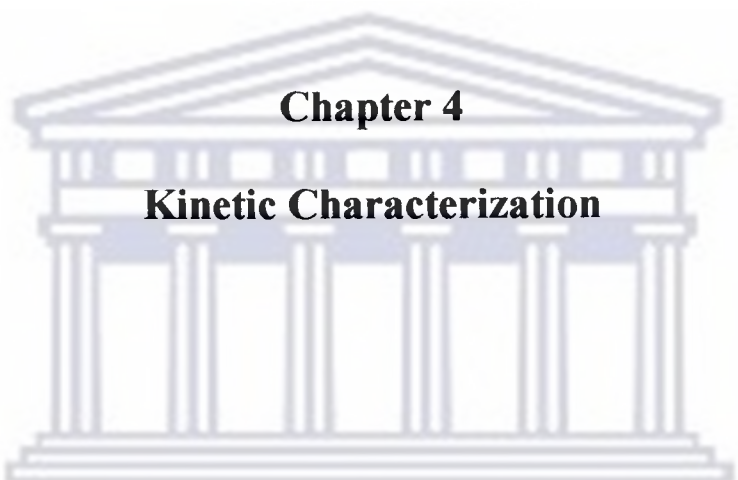
Mass spec data for fractions obtained from all three isolations indicated the presence of a protein with molecular mass of 7 807.8 (+/- 2.2) Da, with the second and third isolations displaying two forms of the protein. Fraction 3-B displayed a form with a tyrosine residue cleaved, while Fraction 4-B displayed a form with an arginine residue cleaved, as determined from the difference in molecular masses of the peaks seen in Figure 3.14. It is interesting to note that tyrosine forms part of the reactive site for chymotrypsin activity, and that none of the proteins exhibited any chymotrypsin inhibitory activity, although homology was indicated with a chymotrypsin/subtilisin inhibitor for one of the forms isolated (molecular mass 7 810 Da). In addition to these proteins, two minor peaks (molecular masses approximately 13 300 Da and 15 500 Da) were also detected by mass spectroscopy analysis. These higher molecular weight proteins could possibly correspond to the first group of double-headed protease inhibitors (Inhibitor I with molecular mass 14 500 Da) described by Odani *et al.* (1986), while the lower molecular eight proteins could correspond to the second group of inhibitors (Inhibitor II with molecular mass 7 000 Da). Alternatively, the higher molecular weight proteins could represent the inhibitors bound to the subtilisin affinity column, since these two peaks are not present in the mass spectroscopy analysis of Fraction 2-A-2 (Figure 3.9a), although proteins which had bound to the subtilisin affinity column did not inhibit trypsin. The fact that mass spectroscopy data for Fractions 3 B and 4 B indicated the presence of the higher molecular weight proteins, in addition to the lower molecular weight proteins, and that the mass spec data for Fraction 2-A-2 indicated the presence of the lower molecular weight proteins only, suggests that both groups of inhibitors can be isolated from the wheat extract. Subjecting the dialysed extract to trypsin affinity chromatography, anion and cation exchange chromatography could possibly purify both groups of inhibitors (Inhibitor I and Inhibitor II).

Sequencing of Fraction 2-H-2 (molecular mass 12 081 Da) proved unsuccessful due to the insufficient quantity of protein. Sequencing of Fractions 2-A-2-B (molecular mass 7 810 Da), 3-B and 4-B were not

conclusive as more than one amino acid was indicated at some of the cycles, while single amino acids were detected at other cycles. The conclusive classification of the three inhibitors was therefore not possible and would require further investigation. CNBr cleavage of Fraction 2-A-2-B and MS-Fit analysis of the resultant peptides obtained, however, revealed that this protein appeared to be homologous to a 8882.3 Da Subtilisin/chymotrypsin CI-1A inhibitor from barley (Accession number P16062), as well as a 6877 Da trypsin inhibitor I precursor from wheat (Accession number Q43667). Since only one major protein of molecular mass 7 807 Da was present in Fractions 3-B and 4-B, it was decided to use these fractions in subsequent kinetic analysis and bioassays.

Chapter 4

Kinetic Characterization



Chapter 4
Kinetic Characterization

4.1 Inhibition Kinetics

The inhibition kinetics of fraction 4-B measured against bovine pancreatic trypsin at 50µg per assay using BAPNA as substrate and at 5µg per assay using TAME as substrate was determined as described in Section 2.3.2.

Results

Dixon plots (1/V vs [I]) derived from experiments using three different substrate concentrations (0.5mM, 0.75mM and 1mM) and four different inhibitor concentrations showed the inhibitor to inhibit the amidase activity of bovine trypsin with a K_i value of $13x10^{-6}$ M (Figure 4.1) and to be a competitive inhibitor. A Cornish-Bowden plot indicated that the esterase activity of bovine trypsin was inhibited with a K_i value of $1x10^{-7}$M (Figure 4.3). Cornish-Bowden (Figure 4.3) and Lineweaver Burke (Figure 4.4) plots determined the inhibitor to be a mixed inhibitor.

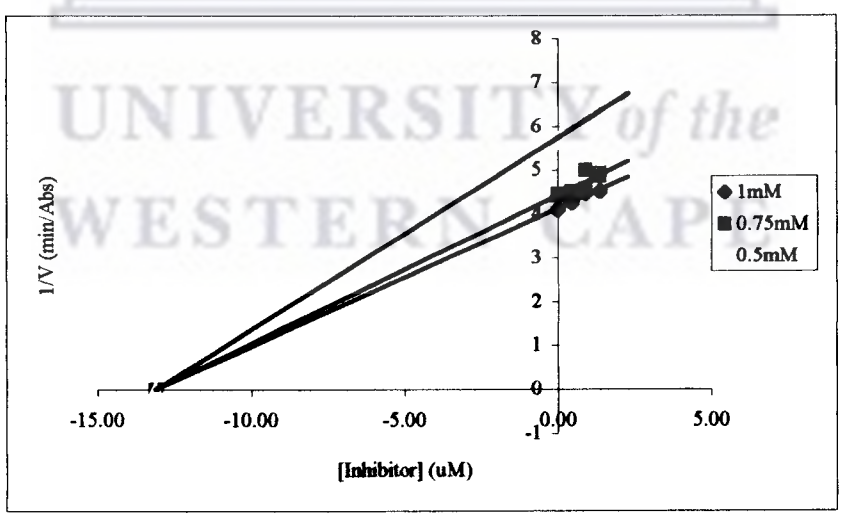


Figure 4.1. A Dixon Plot for the determination of K_i using BAPNA as substrate at concentrations of 0.5mM (R^2= 0.9988). 0.75mM (R^2= 0.994) and 1mM (R^2= 0.9988)

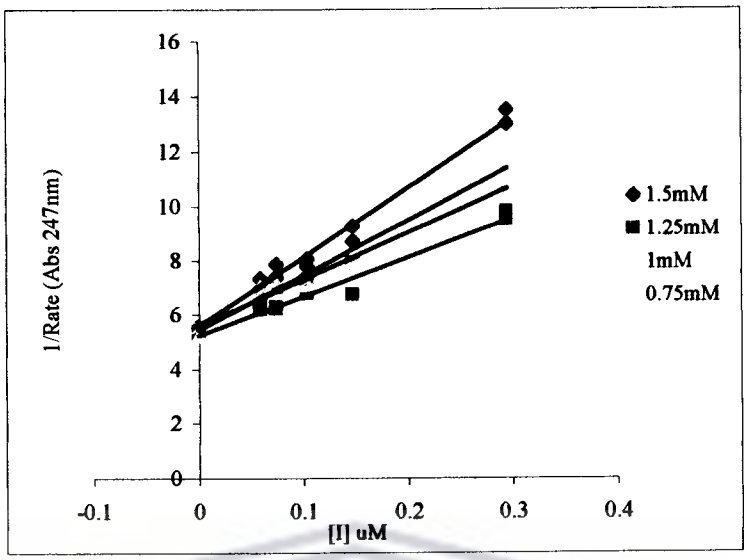


Figure 4.2 A Dixon Plot for the determination of K_i using TAME as substrate at concentrations of 1.5mM (R^2= 0.934), 1.25mM (R^2= 0.967), 1mM (R^2= 0.9597) and 0.75mM (R^2 = 0.9836)

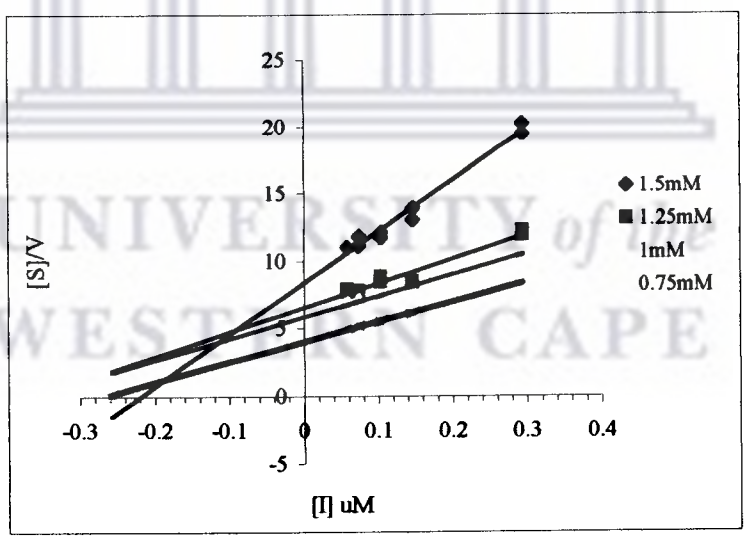


Figure 4.3 A Cornish-Bowden Plot for the determination of K_i using TAME as substrate at concentrations of 1.5mM (R^2 = 0.8571), 1.25mM (R^2= 0.934), 1.mM (R^2= 0.9672) and 0.75mM (R^2= 0.9881)

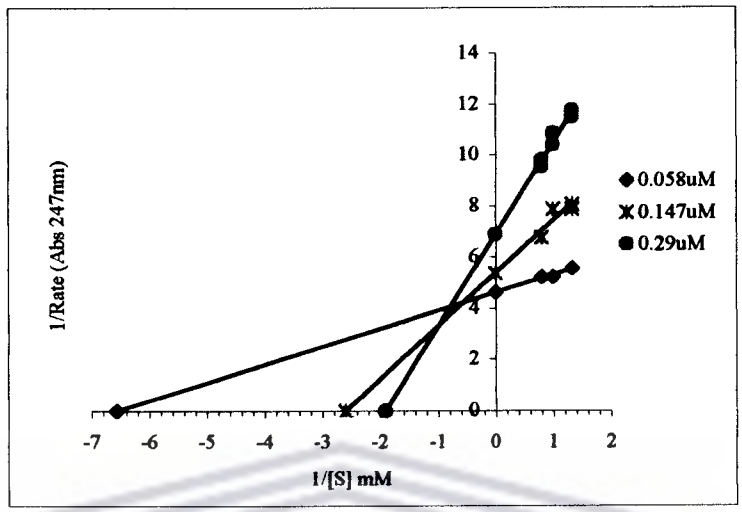


Figure 4.4 A Lineweaver-Burke Plot for the determination of type of inhibition using TAME as substrate and inhibitor concentrations of 0.058uM (R^2=0.9369), 0.147uM (R^2=0.9885), and 0.29uM (R^2 =0.8656)

Table 4.1 Protease inhibitors and reported K_i values

Source	Inhibitor	Substrate	K_i (M)	Reference
Bovine Trypsin	Benzamidine	BAPNA	$1.48x10^{-5}$	Smith *et al.*, 1993
Ostrich Trypsin	Benzamidine	BAPNA	$1.01x10^{-5}$	Smith *et al.*, 1993
Bovine Trypsin	Sh PI		$1.1x10^{-10}$	Delfin *et al.*, 1996
Chymotrypsin	Sh PI	-	$2.3x10^{-9}$	Delfin *et al.*, 1996
Bovine Trypsin	BPTI	-	$6x10^{-14}$	Delfin *et al.*, 1996
Bovine Trypsin	Soybean (Kunitz)	-	10^{-11}	Wu and Whitaker, 1990
Bovine Trypsin	Soybean (BBI)	-	10^{-7} to 10^{-8}	Wu and Whitaker, 1990
Subtilisin	BASI	-	$4x10^{-8}$	Mundy *et al.*, 1983
Trypsin	BBI (Soybean)	-	$5x10^{-9}$	Werner and Wemmer, 1991
Chymotrypsin	BBI		$5.2x10^{-8}$	Werner and Wemmer, 1991
O.nubilalis gut extract: Trypsin-like activity	E-64	BAPNA	$1x10^{-6}$	Novillo *et al.*, 1997
Bovine Trypsin	E-64	BAPNA	$3x10^{-5}$	Novillo *et al.*, 1997
Bovine Trypsin	Benzamidine	BAPNA	10^{-5}	Novillo *et al.*, 1997
Bovine Trypsin	4-aminobenzamidine	BAPNA	10^{-5}	Novillo *et al.*, 1997
Trypsin	SBTI (Kunitz)	S-2222	$3x10^{-12}$	Olson *et al.*, 1995
Trypsin	BPTI (Kunitz)	S-2222	$5.9x10^{-13}$	Olson *et al.*, 1995
Trypsin	α_1 PI (Serpin)	S-2222	$1.4x10^{-11}$	Olson *et al.*, 1995
Trypsin	BWI-1		$1.1x10^{-9}$	Dunaevsky *et al.*, 1995
Chymotrypsin	BWI-1		$6.7x10^{-8}$	Dunaevsky *et al.*, 1995
L.olerecea gut extract	SKTI	SAAPLpNA	$3.1x10^{-7}$	Gatehouse *et al.*, 1999
L.olerecea gut extract	CpTI	SAAPLpNA	$1.4x10^{-6}$	Gatehouse *et al.*, 1999
L.olerecea gut extract	SBBI	SAAPLpNA	$6.6x10^{-8}$	Gatehouse *et al.*, 1999
Trypsin	PAI-1 (Serpin)	S-2222	$1.5x10^{-11}$	Olson *et al.*, 1995

Abbreviations: ShPI- sea anemone PI; BPTI- Bovine Pancreatic Trypsin Inhibitor; BBI- Bowman Birk Inhibitor; BASI- Barley α-amylase/subtilisin Inhibitor; SKTI- Soybean Kunitz Trypsin Inhibitor; CpTI- Cowpea Trypsin Inhibitor; SBBI- Soybean Bowman Birk Inhibitor; E-64- cysteine protease inhibitor; α_1 PI- α_1 Protease Inhibitor; PAI-1- Plasminogen activator inhibitor 1

Discussion

At least seven distinct families of serine protease inhibitors have been described in plants. These proteins have a common mechanism of action, the inhibitor binds to the active site on the enzyme to form a complex with a very low dissociation constant (10^{-7} to 10^{-4} M at neutral pH), thus effectively blocking the active site (Gatehouse *et al.*, 2000). The K_i reported for several wheat α-amylase/trypsin inhibitors is in the order of 10^{-7} to 10^{-8} M, and the K_i for the bifunctional barley α-amylase/subtilisin inhibitor was reported as $4x10^{-8}$ M (Mundy *et al.*, 1983).

The inhibition constant of a trypsin inhibitor from buckwheat seeds was reported as being $1.5x10^{-9}$ M (Gladysheval *et al.*, 1999) and a protein isolated from the seeds of *Medicago scutellata* (which had homology with Bowman Birk trypsin inhibitor family members) was shown to inhibit the catalytic activity of bovine trypsin with an apparent K_d of $1.8x10^{-9}$ (Ceciliani *et al.*, 1997). These values compare well with K_i values obtained by Gariani *et al.* (1999) when they compared 21 synthetic trypsin inhibitor peptides (based on the reactive site loop of the Bowman Birk family) that differed at their P_2'residues (Gariani *et al.*, 1999). The amino acid residues around the reactive site of an inhibitor are usually designated as P_5, P_4, P_3, P_2, P_1, P_1', P_2', P_3', P_4', P_5', where P_1-P_2' is a reactive site peptide bond (Chen *et al.*, 1992). The P_1 site confers the primary specificity; however, members of the Bowman Birk family show variation at the P_2'site and variation of this residue often leads to dramatic changes in K_i values of the inhibitor peptides (Gariani *et al.*, 1999). Peptide P_2' Ile gave the lowest K_i value ($9x10^{-9}$ M) with P_2' Leu and P_2' Arg providing the next lowest K_i value of $1x10^{-6}$M each (Gariani *et al.*, 1999). Inhibition kinetics of the various peptides measured against bovine trypsin were determined using DL-BAPNA as substrate. In the studies conducted by Gariani and Gladysheval where low K_i values were obtained, the common residue at the reactive site was shown to be Arg (Gariani *et al.*, 1999; Gladysheval *et al.*, 1999).

Table 4.1 lists serine protease inhibitors (as well as a cysteine protease inhibitor) from various sources, as well as their reported K_i values. The inhibitors benzamidine and 4-aminobenzamidine are usually regarded as the most effective low molecular weight inhibitors of trypsin, with K_i values of 10^{-5}M. The inhibitor E-64, a common cysteine protease inhibitor, was reported to inhibit the hydrolysis of some trypsin synthetic substrates at K_i values close to those reported for benzamidine and 4-aminobenzamidine, despite its assumed complete specificity for cysteine proteases.

The most prominent member of the Kunitz family of protease inhibitors is bovine pancreatic trypsin inhibitor (BPTI). This protein was shown to have a K_i value of $6x10^{-14}$ M. A protease inhibitor isolated from sea anemone showed sequence homology with inhibitors belonging to the Kunitz family of inhibitors and in particular, similarity to the BPTIs. However, despite this similarity, a K_i value of $1.1x10^{-10}$ M was obtained for the inhibitor using bovine trypsin (Delfin *et al.*, 1996). The high-quality solution structure of the sea anemone PI has an almost identical molecular architecture to BPTI and confirms that this PI belongs to the Kunitz family (Delfin *et al.*, 1996). The K_i value ($1.3x10^{-6}$ M) obtained for the wheat trypsin inhibitor isolated in this study, Fraction 4-B compares favourably with the α-amylase/trypsin and α-amylase/subtilisin inhibitors from the cereal family as well as the Bowman-Birk Trypsin Inhibitor family. The lower K_i values of the Kunitz family of protease inhibitors exclude the isolated inhibitor from this family.

Serine proteinase inhibitors are competitive inhibitors of serine proteinases (Broadway, 1995). Dixon plots using BAPNA as substrate showed the inhibitor to be a competitive inhibitor of trypsin. However, a K_i could not be determined from a Dixon plot derived from experiments using TAME as substrate (Figure 4.2). It was therefore decided to construct a Cornish-Bowden plot (Figure 4.3) from experiments using TAME as substrate. This plot showed the inhibitor to be a mixed inhibitor, which was confirmed by the construction of a Lineweaver-Burke plot (Figure 4.4). A number of possibilities may give rise to mixed competitive and non-competitive effects as seen in this case. However, a mixture of fully competitive cases with non-competitive effects is not possible because in the fully

competitive situation the inhibitor binds with only the free enzyme, whereas in all non-competitive situations the inhibitor must combine with both the free enzyme and the enzyme-substrate complex. Mixed effects can therefore only arise from a combination of partially competitive and non-competitive mechanisms (Ferdinand, 1976).

4.2 Determination of the potential insecticidal properties of the wheat trypsin inhibitor

4.2.1 Inhibitory Activity of Trypsin Inhibitors against Insect Gut Proteases

The inhibitory activity of the isolated protease inhibitors against insect gut protease activity was determined by measuring the inhibition of serine protease activity in *H. armigera* gut extract. This was achieved using the fluorescent substrate Z-Arg-AMC as described in Section 2.3.2.5.

Results

Serine protease activity in the gut extract of *H.armigera* was inhibited by both Fraction 2-A-2 and Fraction 3-B (Figures 4.5 and 4.6). Approximately 65% inhibition of insect gut protease activity was observed for Fraction 2-A-2 and Fraction 3-B at 2.95×10^5nM and 6.46×10^5nM, respectively. Percentage control of protease activity obtained for Fraction 2-A-2, when compared to SKTI, compared favourably. However, percentage control of protease activity for Fraction 3-B, did not compare favourably, since compared to SKTI a higher concentration of Fraction 3-B was required to show a similar level control of protease activity.

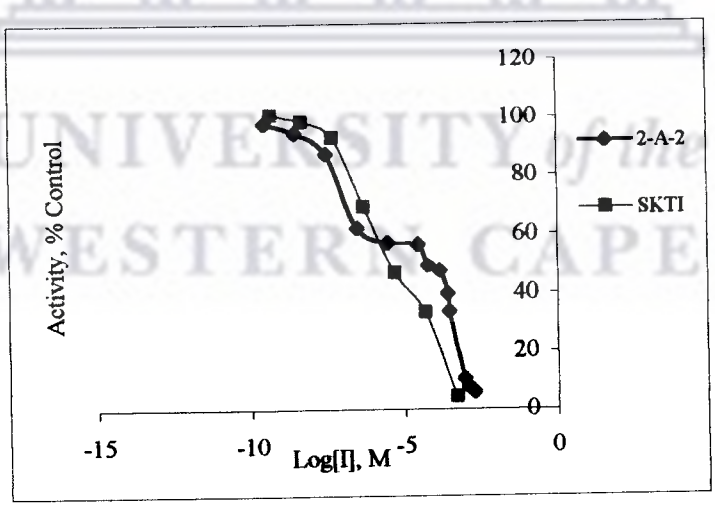


Figure 4.5. Inhibition of *H.armigera* gut serine protease activity by wheat trypsin inhibitor Fraction 2-A-2 (10mM Z-Arg-AMC substrate; microtitre plate fluorimeter protocol)

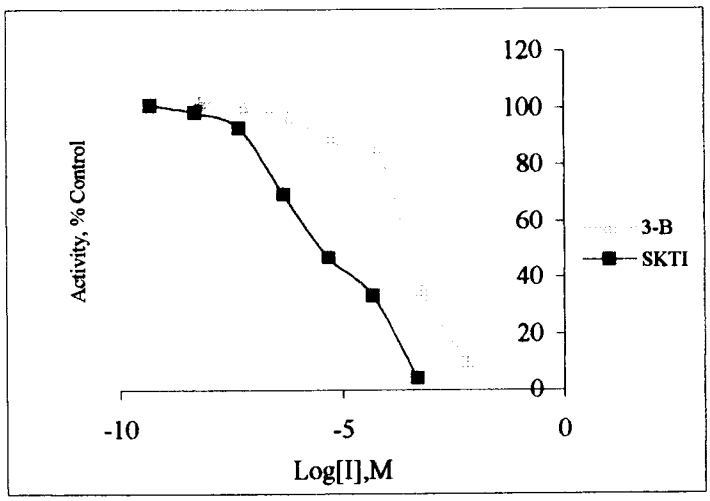


Figure 4.6. Inhibition of *H.armigera* gut serine protease activity by wheat trypsin inhibitor Fraction 3-B (10mM Z-Arg-AMC substrate; microtitre plate fluorimeter protocol)

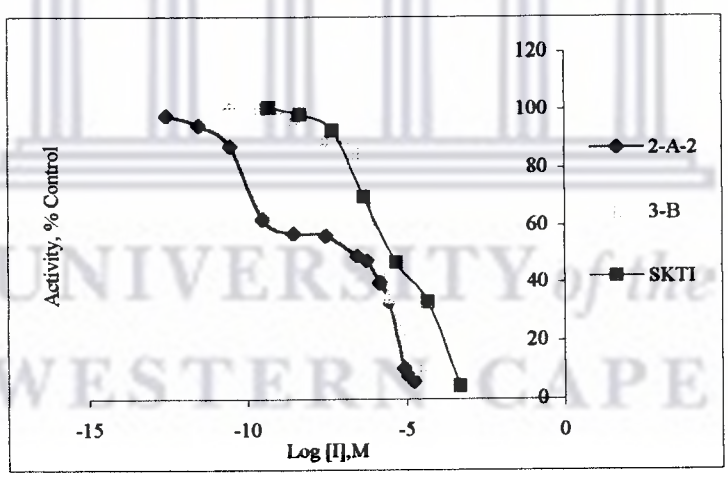


Figure 4.7. Inhibition of *H.armigera* gut serine protease activity by wheat trypsin inhibitor Fractions 2-A-2 and 3-B (10mM Z-Arg-AMC substrate; microtitre plate fluorimeter protocol)

Discussion

Helicoverpa armigera is a Lepidopteran, polyphagous insect pest that feeds on important major crop plants and primarily uses serine proteases for proteolytic digestion (Bown *et al.*,1997; Broadway, 1995). The *in vitro* inhibition of primary digestive proteases is a simple approach to evaluate protease inhibitors for their potential to suppress growth of herbivorous insects (Broadway, 1995). Both trypsin inhibitors tested inhibited serine protease activity in the *H.armigera* gut extract, which is not surprising, since proteolytic activities in the guts of larval *H.armigera* have been shown to be largely due to extracellular serine proteases with high pH optima. Gut extracts of *H. armigera* and the closely related *H.virescens* were found to possess trypsin-like activity (Bown *et al.*, 1997), and inhibition of the proteolytic activity has been demonstrated *in vitro* using protease inhibitors of which soybean Kunitz trypsin inhibitor (SKTI) exhibited the greatest levels of inhibition (Bown *et al.*, 1997). Compared to Fraction 2-A-2 and Fraction 3-B, SKTI exhibited the greatest levels of inhibition (Figure 4.7).

One of the factors determining the potency of a protease inhibitor is its structural compatibility with the proteases in the target organism (Broadway, 1995). The discrepancy in the results obtained when comparing Fraction 2-A-2 and Fraction 3-B could be explained by the fact that although molecular mass of both inhibitors were determined as being 7 810 Da, Fraction 3-B had 2 other proteins (molecular mass of 13 000 Da and 15 000 Da, detected by mass spec analysis) which had co-purified with it during affinity chromatography. Fraction 2-A-2 had been purified from an unbound subtilisin fraction, which was re-chromatographed on a trypsin affinity column and yielded a single protein Fraction 2-A-2 with molecular mass 7 810 Da. It is possible that the proteins present in Fraction 3-B could have played a role in the reduced inhibitory activity of this inhibitor compared to that of Fraction 2-A-2. During trypsin-affinity chromatography, the trypsin inhibitor reacts with the trypsin bound to the matrix. It is a general observation that when naturally occurring trypsin inhibitors interact with trypsin, there is modification of the reactive site and a single peptide bond is cleaved (Mar *et al.*, 1996;

Belozersky *et al.*, 2000). Modification of Fraction 3-B could possibly have occurred, making the inhibitor less effective at binding target enzymes in the insect gut extract. This was also indicated by the two forms detected on mass spec analysis (Figure 3.14) which differed only by the deletion of an arginine (possibly due to the presence of an adjacent lysine or arginine; to be confirmed by sequencing), which could have been cleaved during purification on the trypsin affinity column.

4.3 Antifungal Bioassay

Fraction 4-B at concentrations of 0.25mg/ml, 0.5mg/ml and 1mg/ml was used in the antifungal bioassay against the following fungal isolates *Fusarium verticilliodes* MRC 826, *Fusarium oxysporum* V-101P-E(1)2, *Fusarium pseudograminearum* MRC 6303, *Botrytis cinerea*, *Eutypa lata* (EL 4) and *Penicillium expansum* as described in Section 2.7. The effect of the trypsin inhibitor on germination of conidia from the fungal isolates was determined.

Results

Fraction 4-B showed no effect on the germination of conidia of any of the fungal isolates at any of the concentrations tested (Figure 4.8)

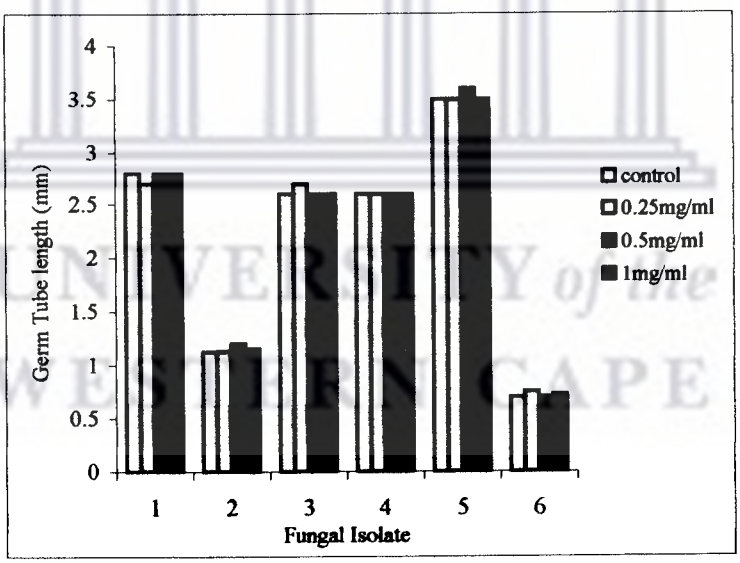


Figure 4.8 Length of germ tubes of fungal isolates tested at 0.25, 0.5 and 1mg/ml of wheat trypsin inhibitor Fraction 4-B. (Fungal Isolates: 1-*Fusarium oxysporum*, 2- *Botrytis cinerea*, 3- *Fusarium verticilliodes*, 4- *Eutypa lata*, 5- *Fusarium pseudograminearum*, 6- *Penicillium expansum*)

Discussion

Antifungal activities have been reported for trypsin inhibitor (TI) proteins from a number of crops including TIs from barley, trypsin and chymotrypsin inhibitors from cabbage, and a 22-kDa TI from corn. However, most were described to be active only against a very limited group of fungi (Chen *et al.*, 1999a). Dunaevsky *et al.* (1998) reported the inhibition of proteases secreted by *Alternaria alternata* and *Fusarium oxysporum* by protease inhibitors from buckwheat at concentrations ranging from 0.35 to 3.1mg/ml. The protease inhibitors also suppressed germination of spores of the fungi tested. Chen *et al.* (1999a) reported the inhibition of plant pathogenic fungi by a corn trypsin inhibitor (14 kDa) over expressed in *E.coli* where conidia were allowed to germinate and grow in the presence of TI at concentrations ranging from 50 to 300µg/ml recombinant trypsin inhibitor. For some isolates they reported that the IC_{50} of the recombinant trypsin inhibitor was much lower than the concentration of native trypsin inhibitor needed to show the inhibitory effect. No inhibition of conidial germination for any of the fungal isolates was detected at concentrations ranging from 0.25mg/ml. Although only 50 to 300µg/ml of recombinant trypsin inhibitor (Chen *et al.*, 1999a) was required to inhibit conidial germination, it is quite possible that much higher concentrations of native plant (wheat) inhibitor may be required. No fungal proteases were available to assay the *in vitro* inhibitory activity of the isolated wheat trypsin inhibitor.

4.4 Cloning and Expression

Mass spectroscopy data, and subsequent trypsin digestion of protein in Fraction 2-H-1 (purified by ion exchange chromatography) indicated its homology to an α-amylase/trypsin inhibitor (accession number P16159). The low yield of the subtilisin inhibitor excluded any further analysis and thus it was decided to attempt cloning and expression of this protein. Amplification of P16159 and cloning into vectors was performed as described in Section 2.6. Cloning into pCR®T7/NT was performed since subcloning from pCR®2.1 into the expression vector pET® 24a was unsuccessful. Induction of protein expression was performed as described in Section 2.6.7.

Results and Discussion

The wheat EST clone obtained from the USDA was successfully used to amplify the P16159 gene. A PCR product (444 bp) was obtained (Figure 4.9). Cloning of the PCR product into pCR® 2.1 was successful as indicated by restriction digestion (Figure 4.10), although sub-cloning into the pET expression vector was unsuccessful. Since sub-cloning into the expression vector did not work, it was decided to clone the PCR product directly into the pCR® T7/NT expression vector. Sequencing results confirmed the identity of the insert and the correct orientation. The induction of proteins with the expected molecular mass was obtained.

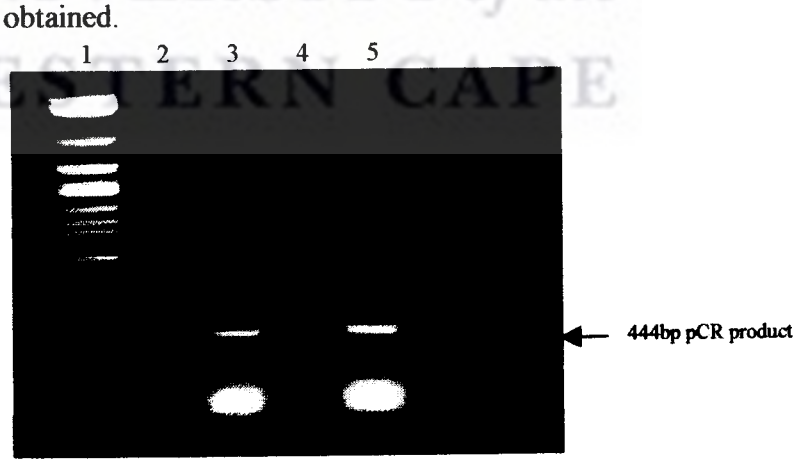


Figure 4.9 PCR amplification of P16159 gene from wheat EST clone. Lane 1: λEco 471; Lanes 3 and 5: 444 bp PCR product

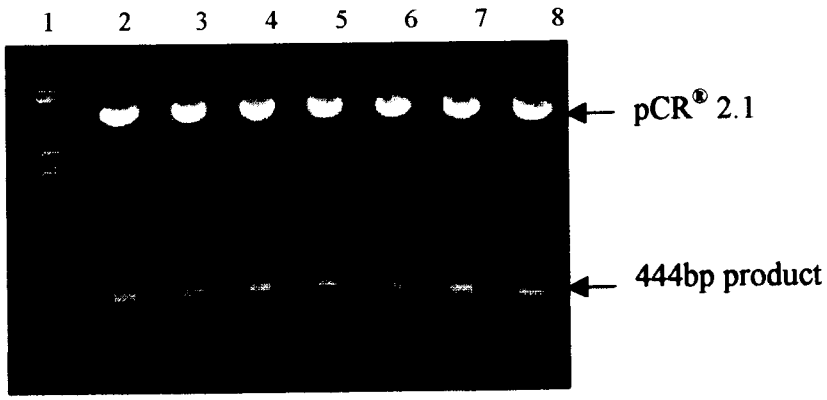


Figure 4.10 Restriction digestion of pCR® 2.1 containing 444bp PCR product. pCR 2.1 was digested with Xho I and NdeI at 37^{0}C for 4 hours. Lane 1: λEco 471; lanes 2-8: digested samples

General Discussion

Triticum aestivum cv. Witwol has been cultivated by resource-poor farmers in the Namaqualand region for the past fifty years, and it is thought that anti-fungal proteins may be involved with its perceived resistance to fungal pathogens (unpublished data). Protease inhibitors are proteins which are induced as part of the plant defense response (Pathogenesis-Related (PR) proteins) and belong to the PR-6 Family (URL5). Protease inhibitors have been isolated from a number of cereal crops, including wheat and barley (Mundy *et al.*, 1984). It was therefore decided to isolate and characterize protease inhibitors from *Triticum aestivum* cv. Witwol and determine the potential of these proteins as antifungal or defensive compounds against herbivorous insects.

Two subtilisin and different forms of a trypsin inhibitor were isolated from *Triticum aestivum* cv. Witwol. Subtilisin affinity chromatography yielded three proteins which all fall within the M_r range for known wheat protease inhibitors. Of these, two proteins were further purified by cation exchange chromatography and found (by mass spectroscopy analysis) to have molecular mass values of 13 282 and 12 081 Da, respectively. The protein with molecular mass of 13 282 Da was found to be homologous to an α-amylase/trypsin inhibitor after trypsin digestion and analysis of the resultant peptide fragments. These proteins did not inhibit trypsin, but it was thought that the elution conditions may be denaturing and therefore affect the inhibitory activity. They were eluted from a subtilisin affinity column however, suggesting that they inhibit subtilisin. No assays were however performed to confirm this. The isolated inhibitors corresponded in size to inhibitors isolated by Mosolov and Shul'gin (1986) and Mundy *et al.* (1984). The trypsin inhibitory activity did not bind to the subtilisin affinity column, and the fact that immobilized subtilisin irreversibly loses its activity at pH values below 4 (Mosolov and Shul'gin, 1986), prompted the need for an alternative affinity column. The unbound subtilisn affinity fraction was then subjected to trypsin affinity and anion exchange chromatography (cation exchange chromatography at this stage proved unsuccessful at purifying any additional proteins). These purification steps yielded a protein with M_r 7 810, which inhibited trypsin

and was shown to be homologous to a subtilisin/chymotrypsin inhibitor from barley, as well as a trypsin inhibitor precursor from wheat. Subsequent isolations from wheat seed, trypsin affinity and anion exchange chromatography yielded a protein with an average molecular mass of 7 807 (+/-2.2) Da which was present (in two separate isolations) in two forms where either an arginine residue or tyrosine residue was cleaved. This protein corresponded in size to group I Inhibitors described by Odani *et al.* (1986). In addition to this major protein, two minor proteins were also shown to be present by mass spectroscopy analysis. Cation exchange chromatography had been excluded since no additional proteins were purified when the trypsin affinity eluent (from the first isolation) was applied to a cation exchange column, but it has been shown that inhibitors not adsorbed to one column may adsorb to another (Dunaevsky *et al.*, 1998). The minor proteins detected by mass spectroscopy analysis may represent the two proteins bound to the subtilisin affinity column although their M_r do not correspond exactly with those detected from the subtilisin column. This is backed up by the absence of these two peaks in the MALDI TOF spectrum of fraction 2-A-2, Figure 3.9(b).

Naturally occurring trypsin inhibitors purified by immobilized trypsin might be proteolytically modified at the reactive sites (Wu and Whitaker, 1990) and the possibility exists that because fractions were not eluted from the trypsin affinity column quickly enough, such modifications may have resulted, and caused the variation in recovery of trypsin inhibitory activity. The use of anhydrotrypsin has been suggested as a means in preventing the modification of inhibitors (Wu and Whitaker, 1990). Since only one major protein (molecular mass 7 807 (+/-2.2) Da) was present in Fractions 3-B and 4-B, these fractions were used in bioassays and kinetic analysis of the protein.

The seven families of serine protease inhibitors in plants have a common mechanism of action and have been shown to be competitive inhibitors of serine proteases (Broadway, 1995). The K_i value (13×10^{-6} M) for the wheat trypsin inhibitor isolated in this study compares favourably with the α-amylase/trypsin and α-amylase/subtilisin inhibitors from the cereal family and the inhibitor was shown to be a competitive inhibitor of trypsin (when using BAPNA as substrate).

Proteolysis is an essential metabolic process required for protein processing and turnover and proteases have been implicated in important developmental processes and are important components in the interaction between plants and other organisms. Protease inhibitors control proteolysis by inhibiting proteases and the regulation of proteases is an integral component of biochemical processes essential for plant growth and development and the plant defense response (Koiwa *et al.*, 1997). The trypsin inhibitors isolated were shown to inhibit the serine protease activity in *H. armigera* gut extract, and compared favourably to levels of inhibition displayed by Soybean Kunitz Trypsin Inhibitor. These results are in agreement with literature which supports the general hypothesis that serine protease inhibitors have the potential to protect plants against herbivorous insects (Broadway, 1995). The serine protease inhibitors Soybean Kunitz Trypsin Inhibitor (SKTI) and Cowpea Trypsin Inhibitor (CpTI) have been shown to effect many Lepidopteran insects, while Coleopterans have been shown to be affected by both the multicystatins from potato and oryzacystatins from rice seed, both of which are cysteine protease inhibitors (Walker *et al.*, 1998). It has been demonstrated that the effective inhibitors of proteolysis in insects *in vitro* are not always effective when the genes encoding these protease inhibitors are expressed in transgenic plants (Gatehouse *et al.*, 1999), as was shown with SKTI being an effective inhibitor of *Heliothis virescens* proteolytic enzymes *in vitro*, while transgenic plants expressing SKTI failed to show any significant levels of resistance towards *H. virescens* (Gatehouse *et al.*, 1999). The inhibitory effect of protease inhibitors on the germination of fungal spores has been demonstrated (Dunaevsky *et al.*, 1998, Chen *et al.*, 1999b) but trypsin inhibitors isolated during this study failed to show any inhibitory activity against any of the fungal isolates tested. The anionic inhibitors isolated from buckwheat suppressed growth and development of pathogenic fungi and inhibited proteases secreted by these fungi, while the cationic inhibitors effectively inhibited some bacterial proteases as well (Belozersky *et al.*, 2000). A trypsin inhibitor over expressed in *E. coli* inhibited the growth of nine fungi tested, which indicates that this protein may have applicability for a broad range of fungal diseases (Chen *et al.*, 1999a). The subtilisin inhibitor (M_r 13 282) was identified

as being homologous to the P16159 gene which encodes an α-amylase/trypsin inhibitor from wheat. Genetic engineering techniques that allow expression of specific foreign genes in microorganisms might afford a route for preparing sufficient homogenous material to carry out studies concerning the protein of interest. It was therefore decided to subclone the P16159 gene from a wheat EST clone into an expression vector. Utilization of the pET expression system proved unsuccessful, although expression of P16159 was achieved using the pCRT7/NT vector. No assays were performed on the expressed protein due to time limitations during the visit to Durham University.

Conclusive classification of the inhibitors isolated will be achieved after sequencing and amino acid analysis of the proteins. The problem of low recovery of inhibitory activity could then be overcome by cloning the genes encoding these protease inhibitors, and over expression of protease inhibitors of interest would then enable further investigation into the antifungal properties, as well as the effect on herbivorous insects.

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