Characterisation of Serine Proteinase Inhibitors in Dry Seeds of Cultivated Pasture Grass Species

A thesis presented in
partial fulfilment of the requirements for
the degree of Master of Philosophy in
Plant Biology and Biotechnology at
Massey University
Palmerston North
New Zealand

Mohammad Tasneem

1994

Abstract

A chymotrypsin inhibitor has been partially purified from *Lolium perenne* cv. Grasslands Ruanui that is stable at pH 3 and after heating at 80°C for 10 minutes (acid/heat stable). The inhibitor had a native molecular weight of *ca.* 20-22 kDa determined using a 2.5 cm x 100 cm Sephadex G-75 column and gel electrophoresis indicated that it may be comprised of two subunits that had molecular weights of *ca.* 11 kDa and 12 kDa. To determine the occurrence of such inhibitors in other grass species, a survey of seeds from several cultivars of pasture grass species was conducted. Seeds of two cultivars of *Festuca arundinaceae*, cv. Grasslands Garland and cv. Grasslands Roa have been found to contain the most potent chymotrypsin inhibitory activity of the species surveyed. Seeds of two cultivars of an economically-important genus, *Lolium*, *L. perenne* cv Grasslands Ruanui and *L. x boucheanum* cv Grasslands Greenstone also exhibited significant chymotrypsin inhibitory activity, and so these four species were studied further. Using a 2.5 cm x 100 cm gel filtration column, chymotrypsin inhibitory activity eluted as two peaks in all four cultivars examined which had native molecular weights of *ca.* 20-22 kDa for peak I and *ca.* 10-12 kDa for peak II. A *ca.* 12 kDa chymotrypsin inhibitor was observed in peak 1 of *F. arundinaceae* cv. Grasslands Garland during gel electrophoresis, and was further purified by ion exchange chromatography. Twelve amino acid residues were sequenced from the N-terminal and the protein was found to be homologous with members of the cereal α-amylase inhibitor family. To purify this activity further, a 10 cm x 100 cm gel filtration column was used. This time trypsin inhibitory activity was also assayed and for all four cultivars, both chymotrypsin and trypsin inhibitory activities co-eluted within two peaks, both with similar molecular weights (peak I, 20-22 kDa and peak II, 10-12 kDa) to that observed previously using the smaller gel filtration column. Gel electrophoresis of peak I from all the four cultivars revealed at least six comparatively lower molecular weight chymotrypsin inhibitors ranging from *ca.* 12 kDa to 3 kDa for the *Festuca* cultivars, and at least two ranging from *ca.* 8 kDa and 12 kDa for the *Lolium* cultivars. However, higher molecular weight trypsin inhibitors were present in Peak I with 5 inhibitors

ranging from *ca.* 12-18 kDa for *Festuca*, and three ranging from *ca.* 12-16 kDa for *Lolium*. Peak II from all the four cultivars contained only one 12 kDa inhibitor band. The 12 kDa inhibitor is active against both trypsin and chymotrypsin. The 12 kDa inhibitor from peak II of *F. arundinaceae* cv. Grasslands Garland was purified further using an anhydro-trypsin affinity chromatography and then to homogeneity by reverse-phase HPLC. Two inhibitory peaks were separated that had dual trypsin/chymotrypsin inhibitory activity and had an identical 20 amino acid residues at the N-terminal. The two inhibitor polypeptides were found to be homologous to members of the barley trypsin inhibitor family, but did not share homology with the *ca.* 12 kDa inhibitor protein purified by ion exchange from gel filtration peak I.

ACKNOWLEDGEMENTS

First of all I would like to thank Almighty ALLAH who enabled me to complete this thesis. With out His help I am nothing. Next is my family who supported and encouraged me morally and financially through out my life. Many thanks to my wife Luna for her patience and support during research and writing up. I would like to thank all my friends in New Zealand and in Pakistan for their help, encouragement and suggestions particularly Tariq Javed, Abdul Khaliq, Manzoor Awan, S. Wajid Hussain, M. Anwar, S.G. Abbas, Babar Mahmood, Shahab, M. Idris. and Dr. M.A. Choudhury.

I would like to thank New Zealand Ministry of External Relation and Trade for tuition fees scholarship, the Miss E.L. Hellaby Trust and Pakistan Banking Council, for financial support.

I wish to thank all the staff of Plant Molecular Genetics Lab, AgResearch Grasslands for their help and cooperation throughout this research project especially Dr. D. W. White for permission to work in his Lab. I also thank all the staff of Department of Plant Biology and Biotechnology, Massey University for their help and cooperation, particularly Mrs Jacky McDonald and Ms E. Nickless.

Thanks are also due to Richard Scott for his cheerful technical help, Dr. Richard Biggs, AgResearch, Palmerston North for the use of anhydro-trypsin affinity column and Dr. W. Laing and Dr. John Christeller, HortResearch, Palmerston North for their assistance with reverse phase HPLC column and technical advice and HortResearch, Palmerston North Library staff for their help with literature search.

I gratefully acknowledge the help of Dr. Nick Elison, AgResearch, Palmerston North, for sequence alignment, Dr. C. Moore, Department of Biochemistry, Massey University, Palmerston North and Dr. David Christie, Department of Biochemistry, University of Auckland for the peptide sequencing, and Photographic Unit, Massey University, Palmerston North for the photography.

Finally I thank to my supervisors Dr. Michael McManus, Dr. Clive Cornford and Dr. David Fountain for their tireless and patient supervision, advice and encouragement.

Table of Contents

List of Figures

List of Tables

LIST OF ABBREVIATIONS

BApNA	N-benzoyl-DL-arginine-p-nitroanalide
BBI	Soybean (Bowman-Birk) trypsin inhibitor
BTI	Barley trypsin inhibitor
BTpNA	N-benzoyl-L-tyrosine-p-nitroanalide
Ca.	Circa
CI-1	Barley chymotrypsin inhibitor 1
CI-2	Barley chymotrypsin inhibitor 2
CMe	Chloroform methanol soluble
CPTI	Cow pea trypsin inhibitor
ELISA	Enzyme-linked immunosorbent assay
FMTI	Foxtail millet trypsin inhibitor
h	Hour
HPLC	High pressure liquid chromatography
kDa	Kilo dalton
Ki	Enzyme inhibition constant
LPC	Leaf protein concentrate
min	Minute
OD	Optical density
P1	Proteinase inhibitor reactive site
PIIF	Proteinase inhibitor inducing factor
PVDF	Polyvinylidene difluoride membrane
RBTI	Rice bran trypsin inhibitor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STI	Soybean (Kunitz) trypsin inhibitor
TFA	Trifluoroacetic acid
Tris	Tris (hydroxymethyl) amino-methane
cv.	Cultivar

Chapter 1

Introduction

Proteinaceous proteinase inhibitors are naturally occurring proteins that can inhibit the catalytic activity of proteinases. They are commonly found in a number of tissues from plants, animals, and insects, as well as in micro-organisms. These inhibitors form reversible stoichiometric protein-protein complexes with specific proteolytic enzymes resulting in the competitive inhibition of their catalytic functions. In most cases their inhibitory activity is active against a single mechanistic class of proteinases only (Richardson, 1977). Since a great deal of information is now known about proteinase inhibitors from both prokaryotes and eukaryotes, discussion on proteinase inhibitors here will mainly be confined to those of plant origin.

In plants, proteinase inhibitors have been isolated and characterised mainly from members of the Leguminosae, Graminae and the Solanacae (Ryan, 1981). More recently, they have been discovered in members of the Cucurbitacae (Nishino *et al.*, 1992) and Cruciferae (Broadway, 1993; Ciciliani *et al.,* 1994). They occur in various plant tissues but are particularly predominant in seeds and tubers. In vegetative tissues, the concentration of proteinase inhibitors is generally low. However, in many plants (eg tomato, potato and alfalfa) wounding of leaves can induce higher levels of proteinase inhibitory activity (Green and Ryan, 1972; 1973; Brown and Ryan, 1984).

Proteinase inhibitors are generally small proteins that range in molecular weight from 4 kDa to 20 kDa. Those inhibitors with a molecular weight exceeding 10 kDa are mostly polymers of two or three protein subunits (Ryan, 1981). A few inhibitors are known to inhibit endogenous proteinase inhibitors in seeds (Shain and Mayer, 1968; Kirsi and Mikola, 1971), but in most cases inhibition of plant proteinases has not been found. Their activity, however, arises from the strong inhibition of the animal proteinases, particularly those of the digestive system i.e. trypsin and chymotrypsin, as well as proteinases of micro-organisms and insects (Richardson, 1977).

1.1 Proteinases and their Inhibitor Groups

Before going into a discussion of proteinase inhibitors, the nomenclature and groups of proteinases will be discussed here because proteinase inhibitors are designated by virtue of the corresponding proteinase group they inhibit. Therefore, information on the classification of proteinases helps with a better understanding of classification of the proteinase inhibitors.

According to recent nomenclature (Barret, 1986) peptidases (=proteases) are enzymes that degrade all types of proteins. These can be subdivided further into endopeptidases and exopeptidases. Proteinases are endopeptidase-type proteases. Proteinases from all living organisms can be further divided into four groups depending on their catalytic mechanisms (Barret, 1986).

1.1.1 Serine proteinases

The serine proteinases have been extensively studied and form the largest group of proteinases which is ubiquitous and diverse in both plants and animals as well as in bacteria. Two super families (the chymotrypsin super family and subtilisin super family) exist in this group. The chymotrypsin type proteinases are found in both prokaryotes and eukaryotes, but subtilisin type proteinases are present in bacteria only. The active site of all the proteinases in this group contains two specific amino acids, serine and histidine (Barret, 1986).

1.1.2 Cysteine proteinases

The second largest and widespread group of proteinases are the cysteine proteinases. Proteinases from this group are recognised by the presence of two amino acids, cysteine and histidine, at the active site (Barret, 1986).

1.1.3 Metallo-proteinases

The metallo-proteinases are common in both eukaryotes and prokaryotes. These have a metal at the active site. In most of the cases zinc has been recognised at the active site (Barret, 1986).

1.1.4 Aspartic proteinases

This group is identified by the presence of aspartic acid at the active site . Aspartic proteinases have been found mainly in eukaryotes. These proteinases are generally active in acidic conditions with the optimum pH from pH 3.5 to pH 5.5, and because of this they have been called acidic proteinases previously (Barret, 1986).

Inhibitors of all of these types of enzymes have been reported from plants and micro-organisms and so they are grouped according to the corresponding proteinase group they inhibit (Richardson, 1977). Therefore, four inhibitor groups designated serine proteinase inhibitors, cysteine proteinase inhibitors, metallo-proteinase inhibitors and aspartic proteinase inhibitors are found. Inhibitors of each proteinase are further classified into different families. The criteria for this classification are based on amino acid sequence homology and primary structure, and the amino acid present at the reactive site of the inhibitor protein (Laskowski, 1986; Ryan, 1990).

The majority of proteinase inhibitors identified and characterized so far are inhibitors of serine proteinases. During the last decade a large number of inhibitors of cysteine proteinases have also been reported and characterized. However, only a few inhibitors of metallo proteinases or acid proteinases have been found in plants to date (Laskowski and Kato,1980; Richardson, 1977; Barret, 1987). Inhibitors of each of these groups will be discussed in this chapter, although most attention will be given to serine proteinase inhibitors.

1.2 Serine Proteinase Inhibitors

Most of the inhibitors found so far in plants inhibit the serine proteinases of animals, insects and micro-organisms. These inhibitors have been found primarily in storage tissues such as seeds and tubers. In these tissues, proteinase inhibitors represent several percent of the total protein content of the organ. Serine proteinase inhibitors have also been found in other vegetative tissues but generally at a lower concentration than in storage organs.

By 1981, more than 100 serine proteinase inhibitors had been reported (Ryan, 1981). Some of these have been well characterized and sequenced and so classified in the already existing families (Laskowski, 1986) or established a new family (Menagatti *et al.*, 1992; Ciciliani *et al.*, 1994). Other inhibitors need further characterization and amino acid sequence determination before these can be assigned to a family.

1.2.1 Mechanism of action

Serine proteinase inhibitors contain one or more reactive sites (P1) that interact with the active site (specific amino acid) of the enzyme. This reaction results in a stable complex of enzyme and inhibitor and no hydrolysis of the peptide bond of the inhibitor occurs (Ryan, 1981).

Inhibitors can have either one or two reactive sites. An inhibitor that has a single reactive site is called single headed. The single reactive site of the inhibitor may be specific for only one serine proteinase (eg trypsin). An inhibitor with two reactive sites is referred to as double headed. The two reactive sites of double headed inhibitors may be specific for only one serine proteinase (eg trypsin in the case of an inhibitor from black eyed peas, *Vigna sinensis*; Gennis and Cantor, 1976) or two independent serine proteinases (eg trypsin and chymotrypsin for the Bowman-Birk soybean inhibitor; Odani and Ikenaka, 1972).

To identify the specific amino acid that is present at the reactive site of the inhibitor, and responsible for inhibitory activity, the protein is incubated for usually more than 24 h with a catalytic amount of the respective proteinase

(trypsin for a trypsin inhibitory site and chymotrypsin for a chymotrypsin inhibitory site) at pH 2 to pH 5. The incubation results in hydrolysis of the reactive site peptide bond, yielding two or three modified but still active inhibitor fragments, depending on the number of the reactive sites for that proteinase. These fragments can then be isolated by gel chromatography. When the amino acid present at newly formed carboxyl-terminus of the inhibitor fragment is removed by using carboxypeptidase A or B, the inhibitory activity is lost. This amino acid is then identified by determining amino acid sequence of the newly formed carboxyl-terinus (Ozawa and Laskowski, 1966; Richardson, 1981) and represents the reactive site of the inhibitor. In general, the amino acid at the N-terminus of the reactive site bond does not seem to recognize the respective enzyme (Kowalski and Laskowski, 1972).

Trypsin inhibitors have predominantly two types of amino acids at their reactive sites. The first is arginine (arginine type) at the reactive site (P1), while second is lysine (lysine type) at P1. Both of these residues are recognised by the trypsin or trypsin-like enzymes. Chymotrypsin inhibitors either have tryptophan, phenylalanine, tyrosine, methionine or leucine in the reactive site. Elastase inhibitors contain an alanine residue at P1 (Laskowski and Kato, 1980; Richardson, 1981).

1.2.2 Families of serine proteinase inhibitors

Inhibitors of serine proteinases from both prokaryotes and eukaryotes have been classified into thirteen families (Table 1.1; Laskowski, 1986), out of which six contain inhibitors of plant origin. Recently, a new family of serine proteinase inhibitors from members of the Cruciferae was established (Menagatti *et al.*, 1992; Ceciliani *et al*; 1994).

1.2.2.1 Soybean (Kunitz) trypsin inhibitor family (STI)

The soybean (*Glycine max*; Kunitz) trypsin inhibitor (STI) was the first plant inhibitor identified. Its isolation, characterisation and that of its complex with trypsin was achieved by M. Kunitz (1945; 1946; 1947a; 1947b; 1949), and

Table 1.1 Families of protein inhibitors of serine proteinases

Animals
1. Bovine pancreatic trypsin inhibitor (Kunitz) family
2. Pancreatic secretory trypsin inhibitor (Kazal's) family
3. Ascaris inhibitor family
4. Serpin family (mechanistically different)
5. Hirudin family
Plants
6. Soybean (Kunitz) trypsin inhibitor family (STI)
7. Soybean (Bowman-Birk) proteinase inhibitor family (BBI)
8. Potato I family
9. Potato II family
10. Barley trypsin inhibitor family (BTI)
11. Squash inhibitor family
Microbial
12. Streptomyces subtilisin inhibitor (SSI) family
13. Other families

From Laskowski (1986)

is considered to be one of the major accomplishments in the early period of research on protein inhibitors from plants. Similarly, its amino acid sequence determination (Koide and Ikenak, 1973a; 1973b; Koide *et al.*, 1973) and further studies on its three dimensional structure (Sweet *et al.*, 1974) and its mechanism of interaction with its enzyme (Sealock and Laskowski, 1969; Kowalski and Laskowski, 1976a; 1976b; Baillargeon *et al.*, 1980) has been done, making it the first plant inhibitor to be well characterised.

The soybean (Kunitz) trypsin inhibitor is strongly active against trypsin and only weakly against chymotrypsin. The inhibitor is single headed, has two disulphide bridges (Koide *et al.*, 1973) which, when reduced with thioredoxin or dithiothreithreitol (Jiao *et al.*, 1992) results in inactivation of the inhibitor protein. The STI has 181 amino acid corresponding to a molecular weight of 20 kDa (Koide *et al.*, 1973). The reactive site for both trypsin and chymotrypsin is arginine (Bidlingmeyer *et al.*, 1972).

Inhibitors from winged bean (*Psophocarpis tetragonolobus*; Kortt, 1979), barley (*Hordeum vulgare*; Yoshikawa *et al.*, 1976; Hejgaard *et al.*, 1983; Svendsen *et al.*, 1986) and potato (*Solanum tuberosum*) tubers (Walsh and Twitchell, 1991) have been identified that are homologous to the STI. Recently, new members were reported from winged bean nodules (Manen *et al.*, 1991) and *Canavalia lineata* seeds (Terada *et al.*, 1994a; 1994b; 1994c).

1.2.2.2 Soybean proteinase inhibitor (Bowman-Birk inhibitor) family (BBI)

A second trypsin inhibitor from soybean (*G. max*) was first identified and separated by Bowman (1946). Later, this inhibitor was further characterised by Birk (1963a), and is presently known as the Bowman-Birk inhibitor (BBI). The full amino acid sequence and the primary structure of the BBI has been determined (Odani and Ikenaka, 1972; Odani and Ikenaka, 1973a)

The soybean trypsin inhibitor (BBI) has a molecular weight of 8 kDa with 71 amino acid residues forming seven disulphide bridges (Birk, 1985). These disulphide bridges are important for activity since incubation of the protein with thioredoxin or dithiothreithreitol inactivates the inhibitor (Jiao *et al.*, 1992). The BBI is double headed inhibitor, i.e. it has independent reactive sites for trypsin

and chymotrypsin (Birk, 1985). The double headed nature of BBI was confirmed by Odani and Ikenaka (1973b) who fragmented the molecule with cyanogen bromide and pepsin into the two active fragments, one with trypsin inhibitory activity and the other with chymotrypsin inhibitory activity.

A large number of inhibitors from different plant sources have been classified in to the BBI family (Garcia-Olmedo *et al.*, 1987). Recently members from seeds of faba beans (*Vicia faba*; Asao *et al.*,1991), pea (*Pisum sativum*; Frokiaer *et al.*, 1994), *Canavalia lineata* (Terada *et al.* 1994a; 1994b), wild *Glycine* species (Kollipara and Hymowitz, 1992), rice (*Oryza sativa*) grain (Laskowski and Kato, 1980), wheat germ (Odani *et al.*, 1986) and barley (*H. distichum*) root (Nagasue *et al.*, 1988) have recently been reported. A wound-inducible trypsin inhibitor from alfalfa (*Medicago sativa*; Brown and Ryan, 1984) has also been placed in the BBI family. The inhibitor is induced by wounding (Brown *et al.*, 1985) in a similar manner to members of the potato inhibitor I and inhibitor II families and this will be discussed in the next section.

1.2.2.3 Potato inhibitor I and II

Potato inhibitor I and II have been extensively studied and so will be considered in detail here. Both inhibitor I and II were first discovered in potato (*Solanum tuberosum*) tubers, where they appeared during early tuber formation and continued to accumulate until the tubers are mature (Balls and Ryan, 1963; Ryan *et al.,* 1976). The two inhibitors can account for up to 15% of the soluble proteins of potato tubers (Ryan *et al.*, 1976; Ryan, 1984).

In tissues of members of the Solanacae family, both inhibitor I and II are commonly found together (Plunkett *et al.*, 1982; Pearce *et al.*, 1988) . The inhibitors are wound-inducible (environmentally-regulated) in tomato (*Lycopersicum esculentum*) and potato leaves (Ryan, 1978) and developmentally-regulated in potato tubers (Ryan, 1984) and in fruit of wild tomato species (Pearce *et al.*, 1988; Wingate and Ryan, 1991).

Inhibitor I and inhibitor II are also present in vegetative tissues of potato and tomato but at a low level. However, in leaves of potato and tomato, the two inhibitors accumulate to a high level (more than 2% of the soluble protein)

within 48 hours after insect attacks or other severe wounding and are considered to be part of a defense response against insects (Green and Ryan, 1972; Brown and Ryan, 1984; Graham *et al.*, 1986). The accumulation is initiated by a putative wound hormone called the proteinase inhibitor inducing factor (PIIF; Green and Ryan, 1972; Ryan, 1974; Ryan, 1984). PIIF is produced at the wound site and transported throughout the plant where it signals leaf cells (including unwounded cells) to initiate synthesis and accumulation of both inhibitor I and inhibitor II (Gustafson and Ryan, 1976). Recently, the PIIF has been identified and characterized in tomato leaves and called systemin (Pearce *et al.*, 1991). In response to wounding, the systemin is synthesised as a large precursor protein (prosystemin) containing 200 amino acids and finally processed into a small polypeptide consisting of 18 amino acids which is the active systemin peptide (Pearce *et al.*, 1991; McGuri *et al.*, 1992).

1.2.2.3.1 Potato inhibitor I

Potato inhibitor I is a pentamer with a molecular weight of 41 kDa. Each monomer has a molecular weight of 8.3 kDa, one disulphide bridge and one reactive site that specifically inhibits chymotrypsin with a Ki of about 10^{-9} M (Ryan, 1984). It is stable at low pH and high temperature for several minutes (Melville and Ryan, 1972). At least 10 iso-inhibitors of inhibitor I on the basis of iso-electric points are present in potato tubers (Richardson, 1977). Of those that have been purified, the reactive site is either leucine or methionine (Richardson, 1977).

So far, the complete amino acid sequence of inhibitor I has not been determined from the purified protein. Instead, it has been deduced from cDNA clones in tomato leaves (Graham *et al.*, 1985a) and in potato (Lee *et al.*, 1986; Cleveland *et al.*, 1987). Besides potato and tomato, members of the inhibitor I family have been found in etiolated tobacco (*Nicotiana tobacum*) leaves (Kuo *et al.*, 1984), tobacco callus tissue (Wong *et al.*, 1975), broad bean seeds (*Vicia faba*; Svendsen *et al.,* 1984) and barley (*H. vulgare*) seeds (two chymotrypsin inhibitors designated CI-1 and CI-2; Svendsen *et al.,* 1980; 1982; Jonassen and

Svendsen, 1982), and in a lower animal, the leech (*Hirudo medicinalis*; Seemuller *et al.*, 1980).

1.2.2.3.2 Potato inhibitor II

Inhibitor II is a dimer with a molecular weight of 21 kDa and is composed of two monomers. Each monomer has a molecular weight of 10.5 kDa with five disulphide bridges and two reactive sites (Bryant *et al.,* 1976; Ryan, 1984). Four iso-inhibitors of the inhibitor II have been reported of which three have been characterised and are potent inhibitors of chymotrypsin. However, only two of these iso-inhibitors inhibited trypsin to any extent while the third one had very weak trypsin inhibitory activity (Bryant *et al.,* 1976). For the iso-inhibitors in the first category, the first reactive site (numbering from the amino terminal) is an arginine and inhibits trypsin while second reactive site is a leucine and inhibits chymotrypsin (Graham *et al.,* 1985b; Sanchez-Serrano *et al.,* 1986; Ryan, 1989). Iso-inhibitors in the second category have leucine at both reactive sites and inhibits chymotrypsin only (Keil *et al.,* 1986; McManus *et al.*, 1994c). All the three iso-inhibitors are stable at low pH and high temperature for several minutes (Bryant *et al.,* 1976).

The complete amino acid sequence was not determined for inhibitor II from proteins purified from potato and tomato, but was deduced from the corresponding cDNA clones in tomato (Graham *et al.,* 1985b) and in potato (Keil *et al.,* 1986; Sanchez-Serrano *et al.,* 1986). A high homology was found between the deduced amino sequences of tomato and potato. A high degree of homology was also observed between the inhibitor II from potato and an inhibitor from eggplant (*Solanum melongena* L) (Richardson, 1979), and an auxin-inducible inhibitor from tomato roots (Taylor, 1993; Young *et al.*, 1994).

Small peptides that have trypsin or chymotrypsin inhibitory activity and are related to inhibitor II have also been reported from potato tubers (Hass *et al.,* 1982). These small peptides were proposed to arise by the post-translational processing of inhibitor II protein in potato tubers (Hass *et al.,* 1982). This was confirmed when a single member of the inhibitor II gene family was transformed into tobacco, and PCI-1 was identified, with the mature

inhibitor II protein as a foreign gene products (McManus *et al.*, 1994b). These small peptides are also identical to peptides identified in the stigmas of tobacco flowers (*Nicotiana alata*; Atkinson *et al.*, 1993) and in wounded tobacco (*Nicotiana tobacum*) leaves (Pearce *et al.,* 1993; McManus *et al.*, 1994a). In tobacco stigma, these smaller peptides (five peptides with a molecular weight of 6 kDa) arise from processing of a larger protein (a 42 kDa precursor; Atkinson *et al.*, 1993).

1.2.2.4 Squash trypsin inhibitor family

Inhibitors of serine proteinases have also been isolated and sequenced from members of the Cucurbitacae (Wilusz *et al.,* 1983; Wieczorek *et al.*, 1985). These were not similar to any existing inhibitors families, and so a new family of serine proteinase inhibitors was recognised and designated as the squash trypsin inhibitor family.

These inhibitors are very small, containing 29-32 amino acid residues forming three disulphide bridges and are active against trypsin and activated Hageman factor (Factor XIIa of the intrinsic blood clotting process). The reactive site is arginine. Their molecular weight is about 3 kDa (Wieczorek *et al.,* 1985).

So far, members of this family have been found only in the Cucurbitacae and have been identified from squash (*Cucurbita maxima;* Wilusz *et al.*, 1983), *Momordica repens* (Joubert, 1984), zucchini (*C. pepo*), summer squash (*C. pepo*), cucumber (*Cucumis sativus*; Wieczorek *et al.,* 1985), bitter guard (*Momordica charantia*; Hara *et al.*, 1989), sponge gourd (*Luffa cylindrica*; Hatakeyama *et al.*, 1991), pickling melon (*Cucumis melo*; Nishino *et al.*, 1992), gourd (*Lagenaria leucantha*; Hamato *et al.*, 1992) and bottle gourd (*Lagenaria leucantha*; Matsuo *et al.,* 1992).

1.2.2.5 Barley trypsin inhibitor family (BTI)

A heat stable trypsin inhibitor from barley (*H. vulgare*) seed was first identified and purified by Mikola and Suolinna (1969). Later this inhibitor was identified and characterized from the endosperm of barley (Boisen, 1976). Amino acid sequencing identified a single polypeptide protein with 121 amino acid residues that has molecular weight of 13.3 kDa with arginine as a single reactive site (Odani *et al.*, 1983a).

The amino acid sequence of barley trypsin inhibitor determined by Odani *et al.* (1983a) was not similar to any existing families of proteinase inhibitors, but showed homology with a wheat (*Triticum aestivum*) germ **α**-amylase inhibitor (Kashlan and Richardson, 1981) and so a new family was established called the barley trypsin inhibitor (BTI) family. Later, a trypsin inhibitor from seeds of maize (*Zea mays*; Swartz, *et al.*, 1977; Mahoney *et al.*, 1984), rye (*Secale cereale*; Boisen, and Djurtoft, 1981b; Lyons *et al.*, 1987) and **α**-amylase inhibitors from seeds of sorghum (*Sorghum bicolor*; Bloch and Richardson, 1992) were added to this family. The trypsin inhibitor from maize also inhibits the activated Hageman Factor (Mahoney *et al.*, 1984).

Two bifunctional inhibitors that inhibit two different classes of digestive enzymes, i.e. serine proteinase and **α**-amylases, also belong to the BTI family. The first inhibitor is from Indian finger millet (*Eleusine coracana*) seeds, and inhibits trypsin as well as mammalian pancreatic and salivary **α**-amylases (Shivaraj and Pattabiraman, 1981). It has homology with other members of the BTI family (Campos and Richardson, 1983). The second bifunctional inhibitor is from maize seeds, and inhibits bovine trypsin and **α**-amylase from *Tribolium castaneum* beetle. However, this inhibitor has little homology with other members of BTI family. Moreover, the maize bifunctional inhibitor has a strong homology with thaumatin (a sweet protein) and a TMV-induced (tobacco mosaic virus) protein (Richardson *et al.*, 1987). Because of this homology, the authors suggest that these latter proteins might be involved in the defensive system of plants.

Trypsin inhibitors that are soluble in chloroform and methanol (CMe) in barley endosperm have also been assigned to the BTI family (Barbar *et al.*,

1986a; 1986b; Moralejo *et al.*, 1993). Many cereal **α**-amylase inhibitors have also been reported to be members of the BTI family (Garcia-Olmego *et al.*, 1987)

1.2.2.6 White mustard/oil-rape trypsin/chymotrypsin inhibitor family

Recently serine proteinase inhibitors from seeds of white mustard (*Sinapis alba* L; Menagatti *et al.*, 1992) and oil- rape (*Brassica napus*; Visentin *et al.*, 1992; Ceciliani *et al.* 1994) were reported. The amino acid sequence from these inhibitors show homology with each other but not with any members of existing families. This suggests that these two inhibitors belong to a new family of serine proteinase inhibitors.

The two inhibitors are active against trypsin and chymotrypsin with an enzyme-inhibitor ratio of 1:1 (Ceciliani *et al.*, 1994) suggesting there is a single (shared) reactive site. The two inhibitor also share a similar Ki which for trypsin is 1.6×10^{-10} M (white mustard) and 3×10^{-10} M (oil-rape) while for chymotrypsin is 5×10^{-7} M (white mustard) and 4.1×10^{-7} M (oil-rape; Menagatti *et al.*, 1992; Ceciliani *et al.*, 1994). The inhibitor from white mustard has 63 amino acids with a molecular weight of 7 kDa. (Menagatti *et al.*, 1992). The oil-rape inhibitor has 60 amino acids with a molecular weight of 6.7 kDa. The reactive site is arginine for both trypsin and chymotrypsin (Ceciliani *et al.*, 1994).

1.2.2.7 Serine proteinase inhibitors from *Brassica*

Serine proteinase inhibitors have also been reported from representatives of the genus *Brassica* but further studies are needed before these can be classified into any family. Trypsin inhibitory activity has been found in cabbage *(Brassica oleracea*; Broadway and Missurelli, 1990). The inhibitory activity level in leaves could be increased significantly by wounding (Broadway and Missurelli, 1990). A number of trypsin/chymotrypsin inhibitors have been purified from cabbage leaves that range in molecular weights from 9 kDa to 25 kDa, and with isoelectric points of 4.5 to 5 (Broadway, 1993).

1.3 Inhibitors of Other Proteinases

1.3.1 Cysteine proteinase inhibitors

Inhibitors of the cysteine proteinases that include papain, chymopapain, bromelain and ficin, have been found in animals, plants and micro-organisms (Barret, 1987). In plants, such inhibitors have been found in potato (*Solanum tuberosum*) tubers (inhibitors of papain, chymopapain and ficin; Hoff *et al.,* 1972; Rodis and Hoff, 1984), *Bauhinia pupurea* seeds (inhibitors of papain, ficin and bromelain; Goldstein *et al.,* 1973), pineapple (*Ananus sativas*) stems (inhibitors of bromelain, papain and ficin; Reddy *et al.*, 1975), corn (*Zea mays*; inhibitors of papain, ficin and bromelain; Abe *et al.,* 1980) and rice (*Oryza sativa*; inhibitors of papain and ficin; Abe and Arai, 1985).

Of these inhibitors, those from pineapple stem have been well characterised. At least seven iso-inhibitors are present in pineapple stems with a common molecular weight of 5.6 kDa. The amino acid sequence of one iso-inhibitor (VII) has been determined (Reddy *et al.*, 1975). It is composed of two chains (A and B) comprising 41 (chain A) and 11 (chain B) amino acids respectively. It has been proposed that the two chains, which are linked by disulphide bridges, are generated from a single-chain precursor by excision of a peptide bridge that links the amino-terminal of the A chain to the carboxyl-terminal of the B chain (Reddy *et al.*, 1975).

1.3.2 Metallo-carboxypeptidase inhibitors

A metallo-carboxypeptidase inhibitor was initially discovered as an impurity in the crystalline preparation of potato inhibitor I from potato tubers (Rancour and Ryan, 1968) and was later purified to homogeneity (Ryan *et al.*, 1974). A similar inhibitor was also identified in tomato fruit (Hass and Ryan, 1980a). These inhibitors are polypeptides with a molecular weight of 4.1 kDa and are usually heat-stable. They are strongly active against pancreatic carboxypeptidases A and B and inactive against all plant and most microbial carboxypeptidases (Hass *et al.*, 1981). Inhibition of carboxypeptidase A and B occurs by a competitive mechanism and results in the rapid removal of the

carboxyl-terminal amino acid of the inhibitor, whose cleavage does not lead to the elimination of inhibitory activity (Hass and Ryan, 1980b).

1.3.3 Aspartic proteinase inhibitors

Inhibitors of aspartic proteinases have only been reported from potato tubers. The inhibitor has a molecular weight of 27 kDa and is heat-stable. It is strongly inhibitory to an aspartic proteinase, cathepsin D. This inhibitor has no effect on other aspartic proteinases such as porcine or chicken pepsin, renin or cathepsin E but is active against trypsin and chymotrypsin. The inhibition of cathepsin D is reversible and competitive, with Ki values of 3.8×10^{-7} M (Keilova and Tomasek, 1976a; 1976b).

1.4 Localization of Proteinase Inhibitors in Plant Tissues

Investigation of the localization of proteinase inhibitors in cells has thus far been limited to inhibitor I and II in tomato leaves (Wingate *et al.*, 1991), CI-1 and CI-2 (chymotrypsin inhibitors) in developing barley endosperm (Rasmussen *et al.,* 1990), and the soybean (Kunitz) trypsin inhibitor (STI) in soybean seeds (Horisberger and Tacchini-Vonlanthen, 1983).

Proteinase inhibitor I, using electron microscopy was found in membraneless protein bodies within the vacuoles of tomato leaf cells (Shumway *et al.,* 1970). The presence of inhibitor I in protein bodies was confirmed by using a combination of immunological methods and electron microscope techniques (Shumway *et al.*, 1976). Later, vacuoles were isolated from tomato leaves and found to contain both proteinase inhibitor I and II (Walker-Simmons and Ryan, 1977). Moreover, both inhibitor I and II proteins are synthesised as a preprotein and are finally compartmentalised as a mature protein in the protein bodies of the central vacuole of tomato leaf cells (Nelson and Ryan, 1980) and tomato fruit parenchyma cells (Wingate *et al.*, 1991).

Barley chymotrypsin inhibitor CI-1 and CI-2 were both found to be synthesised on the rough endoplasmatic reticulum which indicates storing of these proteins in vacuoles (Jonassen *et al.*, 1981). Rasmussen *et al.,* (1990)

using protein A-gold immunocytochemistry, demonstrated that barley CI-2 is deposited in the protein bodies of the endosperm cell and localised in the vacuoles. Barley CI-2 antibody labelling was observed in the golgi derived vesicles and surrounding the vacuoles. It seems, therefore, that the golgi complex is involved in the packaging and transport of CI-2 from its site of synthesis to the vacuole.

In soybean seeds, electron microscopy using protein A-gold indicates that the STI, in common with the other inhibitors mentioned above, is localized in the protein bodies and cytoplasm of cotyledonous cells. Moreover, it is also localized in the cell wall and nucleus of cotyledonous cells (Horisberger and Tacchini-Vonlanthen, 1983) suggesting multiple targeting.

1.5 Implication for Animal Health

1.5.1 Nutritional significance

The nutritional significance of plant proteinase inhibitors has been extensively investigated. Increased intake of raw bean and cereals, high in trypsin and chymotrypsin inhibitor activity, stimulates pancreatic juice secretion and causes pancreatic hypertrophy and growth inhibition in rats (Liener and Kakade, 1980). Sosulski *et al.*, (1988) stated that although proteinase inhibitors in cereals seeds are stable at high temperature and low pH (*in vitro*), they seem to be relative weaker inhibitors of proteinases in the digestive system of animals. This suggests that proteinase inhibitors are not harmful to animals if included in their diet.

1.5.2 Allergens

Recently proteinase inhibitors have been suggested to be involved in causing allergy. Izumi *et al.*, (1992) reported that members of barley trypsin inhibitor (BTI) family proteins could be potentially prominent allergens in cereal and legume seeds. This was based on considerable homology between deduced amino acid sequence of a major allergenic protein from rice seeds and a member of the BTI family (Barber *et al.*, 1986a; 1986b; Odani *et al.*, 1983a).

Adachi *et al.*, (1993) confirmed that the nucleotide sequence from two cDNA clones for rice seed allergenic proteins have homology with members of the BTI family of cereals.

1.6 Functions of Proteinase Inhibitors

During the course of identifying and characterising proteinase inhibitors, several different functions have been proposed for these proteins in plants. These include 1) a role as storage proteins 2) to control endogenous proteinases, 3) to protect plant species from herbivory, and 4) to protect plants against insect pests. The first two functions are directly related to the plants own metabolism, while the last two could be part of plants defensive system. All these possible functions are discussed here.

1.6.1 Storage proteins

The role of proteinase inhibitors as storage proteins is supported by their accumulation in seeds (Mikola and Kirsi, 1972) and tubers (Ryan *et al.,* 1968) in large quantities, their subsequent disappearance during germination and sprouting and their richness in sulphur-containing amino acids (Richardson, 1977). The proteinase inhibitors appear to increase in seeds and tubers of many plant genera as they mature (Ryan, 1973; 1981). For example, soybean seeds contain up to 6% (Rackis and Anderson, 1964), barley grains up to 10% (Mikola and Kirsi, 1972) and potato tubers contain up to 15% (Ryan *et al.,* 1968; 1976) of their soluble proteins as proteinase inhibitors.

Using immunological techniques, the presence of chymotrypsin inhibitor I has been studied throughout the life of potato plants (Ryan, 1968; Ryan *et al.,* 1968). The relative levels of chymotrypsin inhibitor I in all tissues of the potato during various growth stages strongly suggests that it is a storage protein (Ryan, 1973). The initially high concentration of inhibitor in the tuber declines rapidly as sprouting starts and growth of young plants begins. Thereafter, the inhibitor accumulates in the new leaves and aerial tissues. However, when tuber formation begins, the inhibitor disappears from the leaves and

accumulates in the new tubers until they mature (Ryan, 1968; Ryan *et al.*, 1968).

Similarly, two inhibitors from soybean, i.e. STI and BBI, disappear during germination (Tan-Wilson *et al.,* 1982). Recent studies suggest that both the STI AND BBI inhibitors are degraded and modified by a protease designated K1 (Papastoitsis and Wilson, 1991). The modification of inhibitors may be the first step that is necessary for other proteases or the same protease to further degrade the protein for utilisation in the growth of the young plant (Papastoitsis and Wilson, 1991).

A significant homology has been observed between the barley trypsin inhibitor and a castor-bean (*Ricinus communis*) storage protein (Odani *et al.*, 1983b). Since there is no evidence that the castor-bean storage protein has proteinase inhibitory activity, it was suggested that inhibitors of plant proteinase may also be storage proteins or form part of a complex of storage proteins (Odani *et al.*, 1983b).

1.6.2 Regulation of endogenous proteinases

Another proposed function for proteinase inhibitors in dormant seeds and tubers appears to be to control the degradation of stored reserves of protein. This possibility is based on observations that a number of plant proteinases are inhibited by inhibitors that occur in the same tissue (Ryan 1973; 1981; Richardson, 1977; 1981; Boisen, 1983).

The inhibitors of endogenous proteinases were first reported in lettuce (*Lactuca sativa*) seeds (Shain and Mayer, 1965). During germination, proteinase activity increased while the inhibitor disappeared (Shain and Mayer, 1968). In another example, a barley seed extract was found to contain three types of proteinase inhibitors, i.e. an inhibitor of barley seed endogenous endopeptidase, an inhibitor of microbial proteinases and an inhibitor of trypsin (Kirsi and Mikola, 1971). Like the lettuce system, the inhibitor of the endogenous proteinase disappears during germination before the proteinase activity begins to increase. The inhibitor of endogenous proteinases is only confined to the resting barley grain, while the other two inhibitors are also found

in vegetative tissues of barley (Kirsi and Mikola, 1971). In seeds of both lettuce and barley, the disappearance of endogenous proteinase inhibitory activity that accompanies increased proteinase activity strongly suggests that the presence of the inhibitor was to control the activity and level of endogenous proteinases in resting seeds. A similar role has been described for endogenous proteinase inhibitors from cowpea (*Vigna unguiculata*) seed (Royer *et al.,* 1974) and rice grain (Horiguchi and Kitagishi, 1971).

In vivo, proteinases and their inhibitors are most probably located in separate compartments. However, *in vitro*, when seeds are crushed to obtain fine flour, the membrane that separates the proteinase from its inhibitor is broken down resulting in mixing of the proteinase and the inhibitor in the extract (Baumgartner and Chrispeels, 1976). To illustrate this point, Baumgartner and Chrispeels (1976), purified an endogenous proteinase inhibitor from mungbean (*Phaseolus aureus*) seeds and concluded that the inhibitor is located in the cytoplasm and not inside protein bodies where the major proteinase is compartmentalised during germination. They suggest that the role of the inhibitor is likely to protect cytoplasmic proteins against degradation by endogenous proteinases if they are released from accidentally ruptured protein bodies.

1.6.3 Plant Protection

Most proteinase inhibitors purified from plants are active against proteinases from micro-organisms, insects, and also from predatory higher animals. Because of this, it is believed that these play a significant role in the plants defensive system. This concept has attracted molecular biologists to isolate genes that code for these inhibitors and use them to make genetically engineered crops with increased insect pest resistance. So far, a number of genes encoding different proteinase inhibitors from different sources have been isolated and used to transform plants.

The use of proteinase inhibitors in this way has arisen from earlier work which showed that plant proteinase inhibitors can inhibit the digestive proteinases of insects, and so retard their growth. In 1947, Mickel and Standish

observed that larvae of certain pests were unable to grow normally on soybean products. These observations led Lipke *et al.* (1954) to study the toxicity of soybean inhibitors on the complete development of *Tribolium confusum*, a common pest of stored grain. The inhibitor was specific to *Tribolium* larval proteinase and was later isolated and shown to completely inhibit proteinases from the larval gut of *T. confusum and T. castaneum* (Birk *et al.*, 1963b). In 1964, Applebaum proposed that proteinase inhibitors in legumes probably evolved as a defense mechanism against insects, and that protein digestion in insects should be considered as an important factor in host plant selection.

Since then, the effects of proteinase inhibitors on insects has been investigated in a number of cases. In barley, a variety that is more resistant to grasshopper attack had a considerably higher anti-chymotrypsin activity in leaves (Weiel and Hapner, 1976). Similarly, seeds of a cowpea (*Vigna unguiculata*) variety, selected for increased resistance against the bruchid beetle *Callosobruchus maculatus* (a storage pest of cowpea seeds) was found to have an elevated level of trypsin inhibitory activity (Gatehouse *et al.*, 1979). When the larvae were fed with the albumin fraction containing the inhibitors at 10% level, they died. However, the albumin fraction containing no inhibitor was not toxic to these insects (Gatehouse *et al.*, 1979). In comparison, the STI and lima bean (*Phaseolus lunatus*) trypsin inhibitor had no effect on these insects (Gatehouse and Boulter, 1983). The presence of the STI in the diet of corn borer inhibited growth and delayed pupation of this insect pest, but maize trypsin inhibitor had no effect (Steffens *et al.*, 1978). From these reports, it can be concluded that serine proteinase inhibitors are not active against proteinases from all the insects. Instead, they have a selective mechanism to inhibit insect proteinases.

A significant advancement in this field that strongly supports the possible involvement of proteinase inhibitors as protective agents against insects came from Ryan's group. Insect attack, wounding or mechanical damage of tomato or potato leaves results in high accumulation (over 2% of the total soluble protein of the leaf) of inhibitor I (Green and Ryan, 1972) and inhibitor II (Ryan, 1984), not only in affected leaves, but also in other parts of the plant. When larvae of beet armyworm (*Spodoptera exigua*) were fed on a diet prepared from

tomato leaves wounded by feeding beet armyworm, the growth rate of the subsequent feeding larvae was reduced by 84% when compared to those fed on diet prepared from unwounded leaves (Broadway *et al.*, 1986). This supports the concept that proteinase inhibitors are potentially toxic proteins and are synthesised and stored in leaves in response to pest attack in tomato leaves (Shumway *et al.*, 1970; 1976; Walker-Simmons and Ryan, 1977).

Supportive evidence of the defensive role of plant proteinase inhibitors was shown by expressing serine proteinase inhibitor genes from different plant sources (cowpea seeds; Hilder *et al.*, 1990, potato tubers; Johnson *et al.*, 1989; McManus *et al.*, 1994c and tomato leaves; Johnson *et al.*, 1989) in tobacco plants under the constitutive CaMV (cauliflower mosaic virus) promoter. Transgenic tobacco plants that accumulated cowpea trypsin inhibitor demonstrated significantly enhanced resistance against a wide range of tobacco insects pests (tobacco budworm; *Heliothis virescens*, corn earworm; *H. zea*, army worm; *Spodoptera littoralis* and tobacco hornworm; *Menduca sexta*). This resistance was also stably inherited to the seventh generation so far (Hilder *et al.*, 1990). Similarly, growth of *Manduca sexta* (tobacco hornworm; Johnson *et al.*, 1989) and *Chrysodeixis eriosoma* larvae (Silver Y moth; McManus *et al.*, 1994c) was retarded when fed on leaves of transgenic tobacco plants that accumulated the inhibitor II protein. These studies show that different serine proteinase inhibitors genes can be used to enhance the resistance of a target plant against selected insect pests.

However, serine proteinase inhibitors (STI and BBI) are not effective against all insects as their addition at high levels in the diet failed to inhibit larval growth of bruchid beetles (*Callosobruchus maculatus*; Gatehouse and Boulter, 1983; Gatehouse *et al.*, 1985) and colorado potato beetles (*Leptinotarsa decemlineata*; Wolfson and Murdock, 1987). However, addition of a synthetic cysteine proteinase inhibitor (E-64) into the diet of colorado potato beetles did have a toxic effect (Wolfson and Murdock, 1987). Moreover, in proteinase inhibitory assays, the STI and BBI did not inhibit the proteinases in the gut of the bruchid beetle while cowpea seed trypsin inhibitor (CPTI) did partially inhibit proteinase activity in this insect. The CPTI also demonstrated some inhibitory activity against cysteine proteinases and the proteinase activity

in guts of bruchid beetles was cysteine like. In addition, a cysteine proteinase inhibitor purified from cowpea seeds completely inhibited proteinase activity from this insect (Gatehouse *et al.,* 1985), confirming that most of the proteolytic activity in guts of bruchid beetles is due to cysteine proteinases. From these studies, it appears that both bruchid beetles and colorado beetles have cysteine proteinases as their important digestive enzymes. This suggests that a combination of serine and cysteine proteinase inhibitors may provide increased resistance for plants against a wider range of insect pests that use either serine or cysteine proteinase separately.

1.7 Serine Proteinase Inhibitors from the Graminae Family

Serine proteinase inhibitors have been purified and characterised from a number of members of the Graminae. In this section a review of the serine proteinase inhibitors reported so far, will be presented.

1.7.1 Serine proteinase inhibitors from cereals

The serine proteinase inhibitors that have been reported from cereals are summarised in Table 1.2. Both trypsin and chymotrypsin inhibitors as well as bifunctional trypsin/α-amylase inhibitors have been found in cereals. These are usually small proteins with molecular weights ranging between 7 kDa to 25 kDa.

On the basis of the amino acid sequence, some inhibitors from cereals have been assigned to already established families while a few are considered as members of new families. However, for others, further characterisation and amino acid sequence determination is needed to classify them into existing or new inhibitor families.

Among the first category of inhibitors found in cereals are the two identical chymotrypsin/subtilisin inhibitors designated as CI-1 and CI-2 from barley (*Hordeum vulgares)* seeds (Kirsi and Mikola, 1971b; Mikola and Soulinna, 1971; Svendsen *et al.*, 1980; Jonassen and Svendsen, 1982; Boisen *et al.*, 1981; Williamson *et al.*, 1988) and dual (trypsin/ chymotrypsin) inhibitors

Table 1.2 Survey of serine proteinase inhibitors from cereals

Species	Source	Designation	Specificity	Molecular weight in kDa	Inhibitor family	Reference
Barley (*Hordeum vulgare)*	Whole grain	CI-1	Chymotrypsin, subtilisin, *Aspergillus* protease	9	Inhibitor-I	Mikola and Soulinna, 1971; Svendsen *et al.*, 1982; Williamon, 1988
	Whole grain	CI-2	Chymotrypsin, subtilisin, *Aspergillus* protease	9.25	Inhibitor-I	Svendsen *et al.*, 1980; Jonassen and Svendsen, 1982; Boisen *et al.*, 1981
	seed endosperm	BTI	Trypsin	13.3	Barley Trypsin Inhibitor (BTI)	Mikola and Soulinna, 1969; Odani *et al.*, 1983a; Barber *et al.*, 1986a; 1986b; Moralejo *et al.*, 1993)
	seed embryo		Trypsin, pronase	16	BBI	Kirsi and Mikola, 1971; Boisen and Djurtoft, 1982
(*H. distichum*)	Seedling root	BRTI	Trypsin	16	BBI	Nagasue *et al.*, 1988
Wheat (*Triticum aestivum*)	Seed	A/T-WI	Trypsin, endogenous α-amylase	23 with 2 subunits of 11		Warchalewski, 1987
	Seed embryo	WGTI	Trypsin	14.5 and 7	BBI	Odani *et al.*, 1986
	seed		Trypsin	9.1		Mossor and Skupin, 1990a; 1990b
	Seed endosperm		Endogenous and animal trypsin	8.5		Poerio *et al.*, 1989
(*T. vulgare*)	Seed endosperm	WTI	Trypsin, weakly chymotrypsin	12.5		Boisen and Djurtoft, 1981a
Rye (*Secale cereale)*	seed endosperm		Trypsin	12.5	BTI	Mikola and Kirsi, 1972; Boisen and Djurtoft, 1981b; Lyons *et al.*, 1987
Triticale	Seed		Trypsin	Between 10 and 14		Madle and Tsen, 1974
	Seed		Chymotrypsin	Between 17 and 20		Madle and Tsen, 1974

Table 2 continued...

Species	Source	Designation	Specificity	Molecular weight in kDa	Inhibitor family	Reference
Rice (*Oryza sativa*)	Flour bran	RBTI	Trypsin	13.3	BBI	Tashiro and Maki, 1980; Tashiro *et al.*, 1987
Maize (*Zea mays*)	seed		Trypsin, Hagman factor	12.5	BTI	Swartz *et al.*, 1977; Mahoney *et al.*, 1984
	seed		Trypsin, Tribolium α-amylase	22	BTI	Richardson *et al.*, 1987
Sorghum (*Sorghum bicolor*)	Whole grain		Trypsin	15		Xavier-Filho, 1974
	Whole grain		Chymotrypsin, elastase, trypsin, subtilisin, pronase	25		Harish-Kumar *et al.*, 1978; 1979
Millet (*Pennisetum*	Seed	TI-1 and TI-2	Trypsin	11		Chandrasekher and Pattabiraman, 1982; Obidairo and Obasuyi, 1991
Foxtail millet (*Eleusine coracana*)		Ragi	Trypsin, α-amylase	13.3	BTI	Shivaraj and Pattabiraman, 1981; Campos and Richardson, 1983
Foxtail millet (*Setaria italica*)	Seed	FMTI-II and III	Trypsin	7.6	BBI	Tashiro *et al.*, 1989; 1990; 1991
Oats (*Avena sativa*)	Seed		Trypsin, chymotrypsin	43.5		Mikola and Kirsi, 1972
Buck wheat (*Fagopyrum esculentum*)	Seed		Trypsin, chymotrypsin	Between 6 and 7	Inhibitor I	Ikeda and Kusano, 1978; 1983; Kiyohora and Iwasaki, 1985a; 1985b
	Seed		Trypsin. chymotrypsin	Between 10 and 11	Inhibitor I	Kiyohora and Iwasaki, 1985a; 1985b
Job's tear (*Coix lacryma-jobi* L)	seed	JBTI	Trypsin, endogenous proteinase	12 and 7	BBI	Ohtsubo, 1985; Ohtsubo *et al.*, 1989; Ary *et al.*, 1988

from buckwheat (*Fagopyrum esculentum*) seeds (Ikeda and Kusano, 1978; 1983; Kiyohora and Iwasaki, 1985a; 1985b). These belong to the inhibitor I family of serine proteinases. Unlike members of this family from potato and tomato, chymotrypsin inhibitor CI-1 and CI-2 in barley vegetative tissues could not be induced after treatment with the proteinase inhibitor inducing factor (PIIF; Kirsi and Mikola, 1977) or after wounding (Weiel and Hapner, 1976). Dual inhibitors from buckwheat have not been investigated in vegetative tissues.

Double headed inhibitors belonging to the BBI family have been purified from barley seed embryo (Boisen and Djurtoft, 1982), barley (*H. distichum*) seedling root (BRTI; Nagasue *et al.* 1988) and rice (*Oryza sativa*) bran (RBTI; Tashiro *et al.*, 1987). The inhibitor from the barley seed embryo is strongly active against trypsin and microbial trypsin-like proteinases and is weakly active against chymotrypsin. It is highly stable to pepsin (for two hours at pH 2) and to high temperature (100°C; Boisen and Djurtoft, 1982). BRTI simultaneously binds two molecules of trypsin. (Nagasue *et al.*, 1988). Similarly, three single headed trypsin inhibitors, one from wheat (*Triticum aestivum*) germ (Odani *et al.*, 1986) and two (FMTI-II and FMTI-III) from foxtail millet (*S. italica*) grain (Tashiro *et al.*, 1990; 1991) have been classified into the BBI family of inhibitors. Another member of the BBI family is a trypsin inhibitor purified from bran of Job's tears (*Coix lacryma-jobi*) seeds. This inhibitor also inhibited an endogenous proteinase (Ohtsubo, 1985; 1989; Ohtsubo *et al.*, 1989; Ary *et al.*, 1988).

A trypsin inhibitor from barley endosperm was isolated (Mikola and Suolinna, 1969) and characterized (Boisen, 1976). The amino acid sequence (Odani *et al.*, 1983a) showed that the inhibitor belongs to a new family that is now called the barley trypsin inhibitor (BTI) family and discussed under that heading (see 1.2.2.4). Members of the BTI family were later reported from finger millet (*E. coracana*; Campos and Richardson, 1983), rye (*Secale cereale*; Lyons *et al.*, 1987) and maize (*Z. mays*; Mahoney *et al.*, 1984; Richardson *et al.*, 1987).

Inhibitors from seeds of sorghum (*Sorghum bicolor*; Xavier-Filho, 1974; Harish-Kumar *et al.*, 1978; 1979), millet (*P. typhoideum*; Chandrasekher and Pattabiraman, 1982; Obidairo and Obasuyi, 1991), oats (*Avena sativa*; Mikola

and Kirsi, 1972), triticale (Madle and Tsen, 1974), wheat (Mossor and Skupin, 1990a; 1990b; Poerio *et al.*, 1989) and a bifunctional inhibitor from wheat that is active against endogenous **α**-amylase and trypsin (Warchalewski, 1987) have been reported, but have not been assigned to any inhibitor family.

1.7.2 Serine proteinase inhibitors from the grasses

Information on the occurrence of proteinase inhibitors in grasses is limited to only three reports. Among these, only two studies (Humphries, 1980; Ross and Detling, 1983) include proteinase inhibitor assays while the third report (Johns, 1986) only includes feeding trails.

Significant trypsin inhibitory activity has been observed in leaves of four prairie grass species that are native to North America (*Agropyron smithii*, *Andropogon gerardii*, *A. scoparius*, and *Bouteloua gracilis*). The trypsin inhibitory activity observed was similar to that observed in tomato leaves (*L. esculentum*). There was more inhibitory activity in young leaves than old leaves of *A. smithii*, but no such difference was observed in *B. gracilis*. The inhibitors were heat labile. The inhibitors could not be induced by wounding in *A. smithii* (Ross and Detling, 1983).

Trypsin inhibitory activity has been investigated in a leaf protein concentrate (LPC; defined as protein extracted and concentrated from leaves for use in animal diets) from various crops including ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*). Both ryegrass and fescue, using a trypsin assay method involving the hydrolysis of casein, were found to contain some trypsin inhibitory activity in a heat precipitated fraction of LPC. Acidity (pH 4) and autoclaving had little effect on the inhibitory activity. However, it was concluded that phenolic compounds which are present in LPC could mainly be responsible for the observed trypsin inhibitory activity in LPC from ryegrass and fescue (Humphries, 1980). The possibility of using LPC prepared from ryegrass (*L. perenne*) and white clover (*Trifolium repens*) leaves as diet for chickens has also been studied (Johns, 1986). When chickens were fed on such diet (LPC prepared from ryegrass and white clover) their growth was retarded and was accompanied by pancreatic hypertrophy and increased

levels of trypsin and chymotrypsin. However, chickens fed on diet containing heat-treated soybean meal were normal (Johns, 1986). Since these abnormalities and growth depression have been observed in chickens fed on raw soybean meal or with the addition of trypsin inhibitors in the diet (Birk, 1985), it was concluded that leaves of ryegrass and white clover might contain trypsin inhibitors (Johns, 1986). However, there is no direct evidence for the presence of trypsin inhibitors in these plants as proteinase inhibitor assays were not performed.

1.8 Objectives

Serine proteinase inhibitors have been found in members of the Leguminosae, Solanacae, Graminae (cereals), Cruciferae and Cucurbitacae where they are mainly related to the plant's defensive response to insect pest attack (Ryan, 1990; Garcai-Olmedo *et al.*, 1987). In the grasses, studies have been limited to North American native grasses (Ross and Datling, 1983).

Pasture grasses are very important world-wide, but particularly so in New Zealand where its economy is mainly based on dairy and sheep farming. In cultivated pasture grasses, there is no direct evidence for the presence of proteinase inhibitors except one report where low trypsin inhibitory activity in concentrated leaf protein of ryegrass and fescue was possibly due to phenolic compounds (Humphries, 1980). In a second report, a conclusion was made that reduced growth of chicken fed on diet including perennial ryegrass and white clover concentrated leaf protein might be due to the presence of trypsin inhibitory activity in these plants (Johns, 1986). Beside these studies, there is no information about the spectrum of proteinase inhibitors that occur in the cultivated pasture grasses particularly in seeds, since seeds are the major source of these inhibitors in other plants.

Given that these proteins commonly occur in seeds of other species, it is reasonable to assume that in grasses, proteinase inhibitors may also be present and might be important in regulating endogenous proteinases during grain filling and subsequent germination or might be involved in protection of seeds against insect predation.

Therefore, the aim of present project is to survey seeds of different genera of pasture grasses for the presence of serine proteinase inhibitors. The objectives were to 1) identify and characterise a spectrum of serine proteinase inhibitors from cultivated pasture grass seeds, 2) to characterise potent serine proteinase inhibitors from these cultivated pasture grass seeds, and 3) to purify one of these potent serine proteinase inhibitors to homogeneity to determine the amino acid sequence at the N-terminal for alignment with other known serine proteinase inhibitors.

Chapter 2

Materials and Methods

2.1 Plant Material

Seeds from the pasture grass cultivars used in this study were obtained from the Margot Ford Forage Germplasm Centre, Grasslands Research Centre, AgResearch, Palmerston North. Seeds were kept in sealed plastic bags at 10°C until required for experiments.

2.2 Chemicals

All chemicals, unless otherwise specified, were purchased from BDH Chemicals New Zealand Limited (Palmerston North, New Zealand) and were of Analar grade or the best grade available.

Bovine chymotrypsin (C-5467), bovine trypsin (T-5785), N-benzoyl-L-tyrosine-p-nitroanalide (BTPNA), N-benzoyl-DL-arginine-p-nitroanalide (BAPNA), acetyl-DL-phenylanine-β-naphthyl ester, and tetrazotized ortho-dianisidine were purchased from Sigma Chemicals Co. (St. Louis, USA).

2.3 Extraction Protocols

2.3.1 Small scale extraction

This method of extraction was used for surveying inhibitory activity in seeds of different grass species. Dry seeds were milled to obtain a fine flour which was then extracted with an extraction buffer [50 mM Tris (hydroxymethyl) amino-methane (Tris)-HCl, pH 7.5, containing 100 mM NaCl] at a ratio of 1 g (gram) fresh weight : 5 ml buffer. Normally 5 g flour was used. The mixture was stirred at 4°C for one hour and the resultant slurry was centrifuged at 20,000 g for 20 min at 4°C. The supernatant (crude extract) was retained and used in inhibitor assays.

2.3.2 Large scale extraction

For the further purification and characterisation of inhibitor proteins, large scale extractions were done. Here, either a 100 g (for chromatography through a 2.5 cm x 100 cm Sephadex G-75 column) or 500 g (for chromatography through a 10 cm x 100 cm Sephadex G-75 column) of flour was extracted in 500 ml or 2,500 ml of extraction buffer respectively and then centrifuged as described in 2.3.1.

2.3.3 Acid and heat treatment

The pH of crude extract was reduced to pH 3 with 1 M HCl, heated at 80°C for 10 min, and then centrifuged at 20,000 g for 20 min at 4°C. The pH of the supernatant was adjusted to pH 8.0 by subjecting the sample to Sephadex G-25 gel chromatography.

2.4 Gel Filtration

2.4.1 Sephadex G-25 gel filtration

Sephadex G-25 (medium grade, Pharmacia-LKB Biotechnology AB, Uppsala, Sweden) was prepared by soaking it in 10 mM Tris-HCl pH 7.5 (Column buffer 1) overnight. Two different methods were used for Sephadex G-25 gel chromatography.

2.4.1.1 Spin column method

To test the effects of acid/heat treatment on yield of proteinase inhibitory activity, small (5 ml capacity volume) Sephadex G-25 columns were used to adjust the pH of the acidified extracts to pH 8.0. Sephadex G-25 columns were poured and placed in 50 ml polycarbonate centrifuge tubes and then centrifuged (IEC, Centra 7, USA) for one minute. The eluent from each tube was discarded and immediately one ml of the acid/heat treated sample was applied to the column and centrifuged again. The eluent obtained was assayed

for protein concentration and chymotrypsin inhibitory activity and a purification table was prepared.

2.4.1.2 Large scale purification method

For large scale purification, a 6 cm x 70 cm Sephadex G-25 column was poured. The bed volume of the column was 1700 ml, flow rate was approximately 15 ml per min and the void volume was calculated to be 600 ml. The acid and heat denatured sample was applied to the column, eluted with column buffer 1 and fractions (15 ml) were collected.

The presence of protein in each fraction was determined and inhibitor assays were performed on fractions that contained protein. Fractions with inhibitor activity were pooled and concentrated using a Filtron Omegacell concentrator (molecular weight cut off 10 kDa; Filtron Technology Corporation, Northborough, Mass., USA). The protein concentration of the concentrated sample was measured and inhibitor assays were performed to check activity prior to gel filtration using Sephadex G-75.

Before re-use, the Sephadex G-25 column was washed with copious amounts of column buffer 1 and then water, before being re-equilibrated with column buffer 1.

2.4.2 Sephadex G-75 gel filtration

Sephadex G-75 (Superfine grade, Pharmacia LKB) was prepared by soaking it in 100 mM Tris-HCl pH 8 (column buffer 2) or 25 mM ammonium bicarbonate (column buffer 3) overnight. Two different size columns were used for Sephadex G-75 gel filtration.

2.4.2.1 Purification I

For the initial purification of inhibitory proteins, a 2.5 cm x 100 cm Sephadex G-75 column was used. Sephadex G-75 was pre-equilibrated with column buffer 2. The bed volume of the column was 500 ml, the flow rate used

was 12 ml/h and the void volume was calculated to be 100 ml (see section 2.4.3). The concentrated sample was centrifuged as described in 2.3.1, applied to the column and eluted with column buffer 2. Fractions (2.5 ml) were collected.

2.4.2.2 Purification II

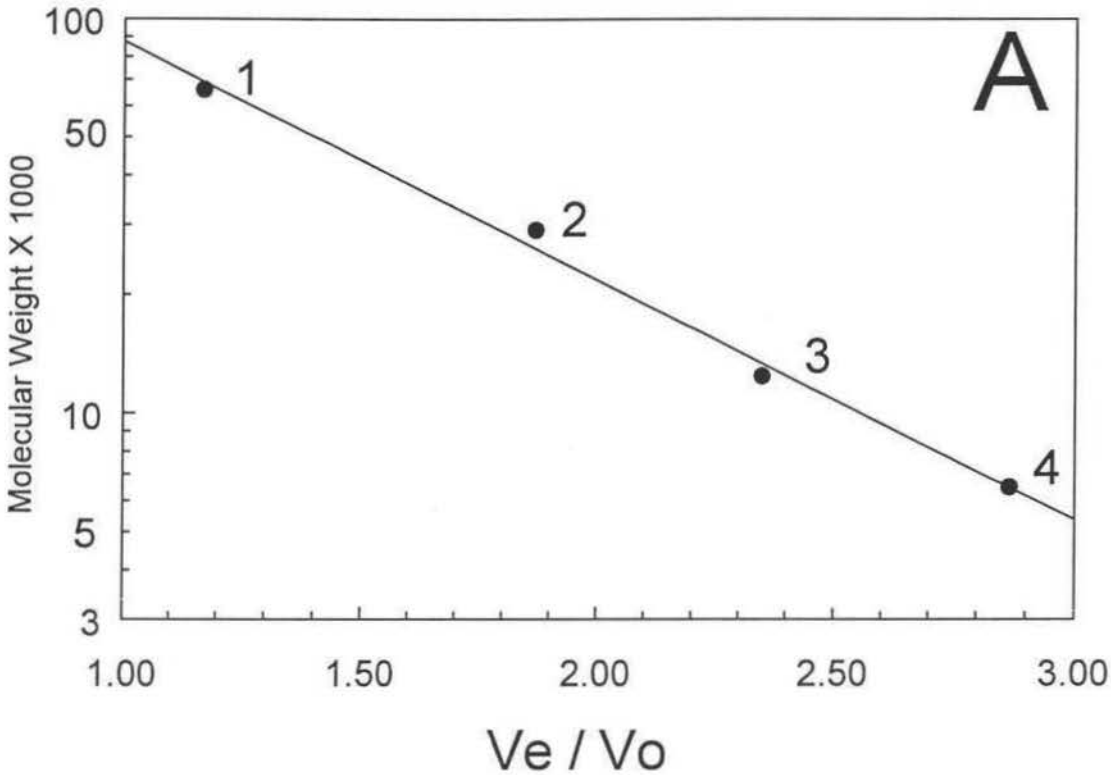
To further resolve proteins, a larger (10 cm x 100 cm) Sephadex G-75 column was used. The bed volume of the column was 1500 ml, flow rate was 30 ml/h and the void volume calculated was 500 ml (see section 2.4.3). Each concentrated sample was centrifuged as described in 2.3.1, applied to the column and eluted with column buffer 3. Fractions (12 ml) were collected after the void volume had been discarded.

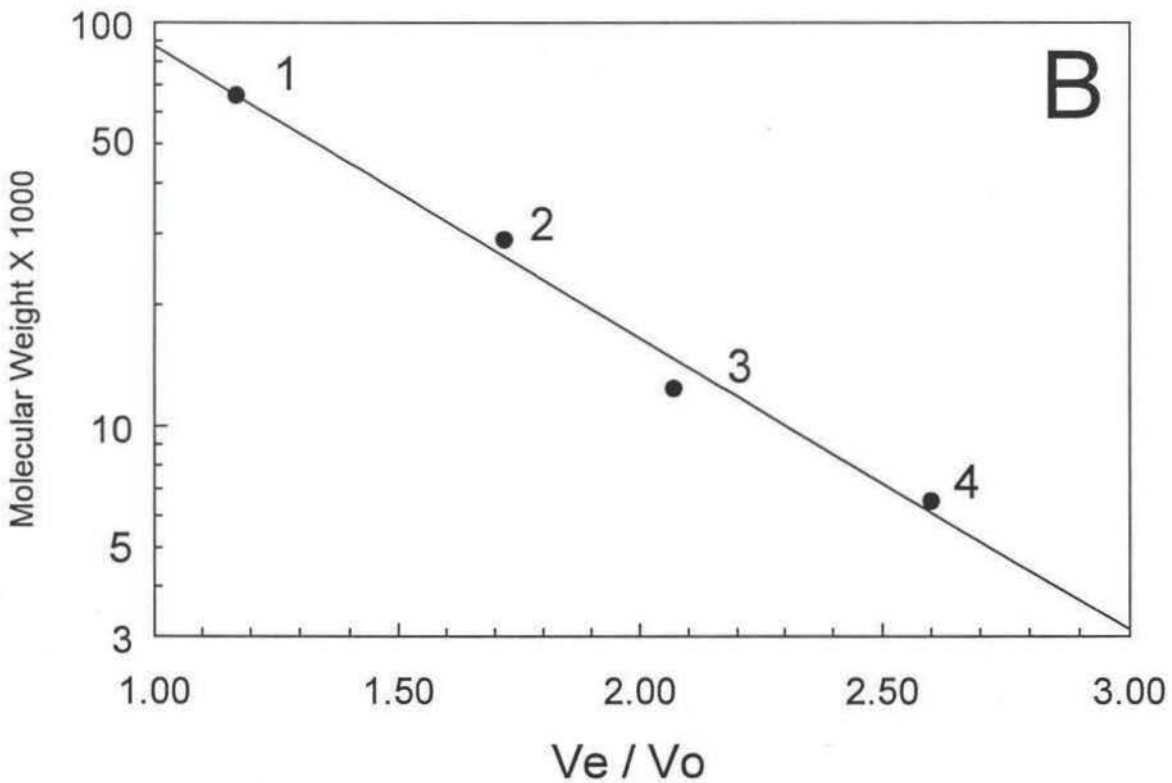
The presence of protein in each column fraction obtained from both columns was determined by measuring the absorbance at 280 nm. Fractions containing protein were assayed for inhibitory activity and fractions containing activity were pooled. Pooled fractions from the smaller G-75 column were concentrated by ultrafiltration using a Filtron omegacell concentrator (Molecular weight cutoff 3 kDa) and stored at 4°C, while pooled fractions from the larger G-75 column were freeze dried and stored at -20°C for subsequent use.

2.4.3 Calibration of Sephadex G-75 column

Before calibration, the void volume of each column was determined with blue dextran (molecular weight of 200 kDa). Sephadex G-75 separates globular proteins within a molecular weight range of 5-75 kDa. Proteins and polysaccharides i.e. blue dextran, in excess of this molecular weight, pass cleanly through the column and the elution volume that corresponds with blue dextran represents the void volume. To calibrate the Sephadex G-75 gel filtration columns for molecular weight determination, a mixture of proteins of known molecular weight (MW-GF-70; Sigma) was used. Calibration curves for two Sephadex G-75 columns are given in Figure 2.1A (smaller column) and Figure 2.1B (larger column).

Figure 2.1 Calibration curves obtained with a mixture of molecular weight standards (MW-GF-70; Sigma) separated through a 2.5 cm x 100 cm Sephadex G-75 column (**A**), a 10 x100 cm column (**B**). 1, bovine albumin (66 kDa); 2, carbonic anhydrase (29 kDa); 3, cytochrome C (14.4 kDa); 4, aprotinin (6.5 kDa).


A
Molecular Weight X 1000
100
50
10
5
3
1
2
3
4
1.00
1.50
2.00
2.50
3.00
Ve / Vo


B
Molecular Weight X 1000
100
50
10
5
3
1
2
3
4
1.00
1.50
2.00
2.50
3.00
Ve / Vo

2.5 Ion Exchange Chromatography

2.5.1 Anion exchange chromatography

Q-Sepharose (Pharmacia LKB) was equilibrated with 100 mM Tris-HCl pH 8.0 (column buffer) and a small column (5 ml) was poured. A 5 mg sample (previously purified with Sephadex G-75 column chromatography) was applied to the column, which was then washed with 5 volumes of column buffer. To elute bound protein, a gradient of 0-0.5 M potassium chloride (KCl; 5 volumes) was used. Fractions (1.5 ml) were collected during loading the sample, washing off unbound proteins and elution of bound protein, and then assayed for chymotrypsin inhibition.

2.5.2 Cation exchange chromatography

A small (5 ml) column of S-Sepharose (Pharmacia LKB) was prepared, after pre-equilibrating the gel with 25 mM sodium acetate pH 4.5 (column buffer). A 10 mg sample (previously purified with Sephadex G-75 column chromatography) was dialysed overnight against column buffer and then loaded onto the column. Fractions (1.5 ml) collected during sample loading and subsequent washing with 5 volumes of column buffer were assayed for chymotrypsin inhibition. To elute bound inhibitor from the column, a gradient of 0-0.5 M KCl was used. Fractions (1.5 ml) were collected and assayed for chymotrypsin inhibition.

Fractions containing chymotrypsin inhibitory activity were pooled, dialysed against 100 mM Tris-HCl pH 8.0 and concentrated using a Filtron Omegacell concentrator (Molecular weight cutoff: 10 kDa). The concentrated samples were used for gel electrophoresis and either stained for protein, proteinase activity or transferred to PVDF membrane using the mini blotting system described below. The inhibitor bands transferred to PVDF membrane were sent to Auckland for N-terminal sequencing (see 2.6.5).

2.5.3 Anhydro-trypsin affinity column chromatography

An Affigel (BIO-RAD Laboratories) 10-anhydro-trypsin column was prepared and kindly supplied by Dr. Richard Biggs, AgResearch Grasslands, Palmerston North, New Zealand.

A freeze-dried sample (10 mg; after Sephadex G-75 column chromatography) was dissolved in column buffer comprising 50 mM Tris-HCl, pH 8.0, 60 mM $CaCl_2$ and 50 mM NaCl and applied to the anhydro-trypsin affinity column. The sample was re-cycled through the column twice and then the column thoroughly washed with column buffer. Bound proteins were eluted with five volumes of elution buffer comprising 10 mM HCl, 500 mM NaCl and 10 mM $CaCl_2$ and 1.5 ml fractions collected. One M Tris-HCl, pH 11.0 was added in each fraction to adjust the pH to 8.0. Each fraction was assayed for the presence of protein and proteinase inhibitory activity. Fractions containing activity were pooled and concentrated using a Filtron concentrator. The concentrated sample was diluted with 25 mM ammonium bicarbonate buffer, and concentrated again. This process was repeated 3 times and finally the sample was freeze-dried and stored at -20°C for subsequent use in SDS-PAGE or reverse-phase column chromatography.

2.5.4 Reverse-phase column chromatography

The affinity purified sample was resuspended in 0.1% (v/v) trifluoroacetic acid (TFA) and loaded onto a 1.0 cm x 30.0 cm Vydac C-18 column (Alltech, Deerfield, Ill.,USA). A linear gradient [10% (v/v) acetonitrile, 0.1%(v/v) TFA to 60% (v/v) acetonitrile, 0.1% (v/v) TFA] was used to elute proteins. The flow rate was 1 ml/min. One ml fractions were collected, and assayed for trypsin and chymotrypsin inhibitory activity. Active fractions were pooled and freeze-dried.

To achieve a better separation, reverse-phase chromatography was repeated using the freeze-dried samples obtained from the reverse-phase separation described previously. To elute proteins, a gradient of acetonitrile [35% (v/v) acetonitrile in 0.1% (v/v) TFA to 48% (v/v) acetonitrile in 0.1% (v/v) TFA] was used. Two peaks of activity were observed in the eluate and these

were freeze-dried separately. To check their purity, both peaks were re-chromatographed separately using the 35% (v/v) acetonitrile in 0.1% (v/v) TFA to 48% (v/v) acetonitrile in 0.1% (v/v) TFA gradient conditions.

2.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

2.6.1 SDS-PAGE

All samples used for SDS-PAGE were reduced and denatured with loading buffer. The loading buffer consisted of 50 mM Tris-HCl, pH 6.8, containing 2.5% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. The mixture was heated at 100°C for three minutes and centrifuged at 13,000 g for 5 min prior to being separated through the polyacrylamide gel. Electrophoresis was carried out essentially as described by Laemmli (1973). Normally a fixed (17.5%; c=2.7%) or a gradient (10-20%; c=2.7%) polyacrylamide gel (20 x 16 cm) containing 0.1% (w/v) SDS was run at 25 mA constant current at room temperature for 6-8 hours. Polyacrylamide gels were used for protein staining or proteinase inhibitory activity staining.

2.6.2 Minigels

Using similar conditions to those described above, samples were separated through minigels (BioRad MiniProtean II System, 10 x 10 cm, 0.5 mm thick) containing 17.5% (c=2.7%) polyacrylamide, at 200 V constant current for 40 min. Normally, separation of samples was performed as either two identical gels or one gel with two identical halves. After electrophoresis, gels were stained for either chymotrypsin/trypsin activity or protein, or electroblotted on to PVDF membrane for N-terminal amino acids sequencing (see 2.7.1).

2.6.3 Protein staining

On conclusion of electrophoresis, gels were stained for at least 2 h at room temperature in 0.5% (w/v) Coomassie brilliant blue R in 50% (v/v) methanol, 10% (v/v) acetic acid. The gels were then destained in 30% (v/v) ethanol.

2.6.4 Proteinase inhibition activity staining

Following electrophoresis, the gel was stained for serine proteinase inhibitory activity to locate the inhibitor band(s) by the procedure described by Xaliev-Filho and De Azevedo-Moreira (1978). Firstly, the gel was fixed by incubating it in a fixative solution (7.5% (v/v) acetic acid, 5% (v/v) methanol) for two hours at room temperature. The gel was rinsed with water six times and then equilibrated with 100 mM phosphate buffer, pH 7.4 for two hours at room temperature. Proteinase [bovine chymotrypsin solution (0.05 mg/ml) or bovine trypsin solution (0.1 mg/ml) in the phosphate buffer] was added and the gel was incubated for 30 minutes at 37°C. After rinsing the gel with water six times, the gel was incubated again for 30 minutes at 37°C without any solution. Proteinase inhibitory activity was detected by adding freshly prepared substrate mixture for 30 minutes at room temperature. The substrate consisted of 0.25% (w/v) acetyl-DL-phenylanine-β-naphthyl ester in 10% (v/v) dimethylformamide and 0.5%(w/v) tetrazotized ortho-dianisidine in 100 mM phosphate buffer pH 7.4. After 30 minutes, the substrate was discarded and the gel was rinsed with and kept in fixative solution. The bands containing proteinase inhibitory activity were clear against an intense pink background.

2.6.5 Electroblotting on polyvinylidene difluoride membrane (PVDF) for sequencing

Electroblotting of samples was performed according to the method described by Matsudaira (1987). After electrophoresis, the gel was soaked in transfer buffer (10 mM 3-[cycohexylamino]-1-propanesulfonic acid, 10% [v/v]

methanol, pH 11) for 5 min to reduce the amount of Tris and glycine. A PVDF membrane was rinsed with methanol and kept in transfer buffer. The gel was then placed between a sheet of PVDF membrane and two sheets of blotting paper and assembled into a blotting apparatus and electroeluted (from gel onto PVDF membrane) for 25 min at 0.5 A in the transfer buffer. After transfer, the PVDF membrane was washed with water, stained with 0.1% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol for 5 min and then destained in 50% (v/v) methanol, 10% (v/v) acetic acid for 10 min at room temperature. Finally, the membrane was rinsed with water, air dried and sent for sequencing to Auckland (see 2.7.1).

2.7 Sequencing and Identity with other Proteins

2.7.1 N-Terminal amino acid sequencing

N-terminal amino acid sequencing of the inhibitor purified by ion exchange and blotted on PVDF membrane was performed by Dr. D. Christie, Department of Biochemistry, University of Auckland, Auckland, New Zealand.

Determination of the N-terminal amino acids sequence of the dual trypsin/chymotrypsin inhibitors obtained after reverse-phase purification, was performed by Dr. C. Moore, Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand, on an Applied Biosystems Model 476A Protein Sequencer (Applied Biosystems, Foster City, California, USA), using the standard protocol for Edman chemistry supplied by the manufacturer.

2.7.2 Sequence homology search

The sequence of the inhibitor proteins was compared with other known protein sequences by Dr. Nick Elison, Agresearch Grasslands, Palmerston North, New Zealand, using two amino acid databases ["Blast-P" (Altschul *et al.*, 1990) and "Fast-A" (Pearson and Lipman, 1988)]. Comparison of the sequence from inhibitors purified here was performed using a computer programme "Align" (Scientific and Educational Software, USA).

2.8 Assays

2.8.1 Protein assay

Protein was assayed using the Bio-Rad protein assay kit based on the dye-binding assay of Bradford (1976).

Two variations of the assay were used

2.8.1.1 Standard assay

Sample (200 μl of an appropriate dilution) was made up to 800 μl with water and 200 μl Bio-Rad dye reagent concentrate was added. The absorbance of the mixture at 595 nm was recorded using CE 272 Readout Ultraviolet Spectrometer (Cecil Instruments Limited Cambridge, England). The OD reading was converted to protein concentration using the following formula.

$$\frac{a \times b}{c}$$

where

a = Protein dilution (1:100)

b = μg protein /cuvette (from a standard curve constructed against potato proteinase inhibitor II. This was prepared by Mr. Richard Scott, AgResearch Grasslands, Palmerston North).

c = μl of protein dilution added to cuvette.

2.8.1.2 Column fractions

G-25 fractions were assayed for the presence of protein by adding 80 μl from each fraction and 80 μl water to a 96 well micro-titre plate (A/S Nunc InterMed, Roskilde, Denmark) and mixing it with 40 μl of Bio-Rad dye reagent concentrate. The absorbance of the mixture was read at 595 nm using a Model 3550 microplate reader (Bio-Rad Laboratories Ltd, Hercules, California, USA). Fractions that were positive for protein were used in proteinase inhibitor assays, while other fractions were discarded.

The presence of protein in all other column eluates was determined by measuring absorbance at 280 nm using a CE 272 Linear Readout Ultraviolet Spectrometer with the appropriate column buffer as blank.

2.8.2 Serine proteinase inhibitor (Pi) assays

Assays were performed in 96-well micro-titre plates. Different dilutions of extract were made in 100 mM Tris-HCl, pH 8.0 (assay buffer) and aliquots (200 μl) placed in individual micro-titre wells while assay buffer (225 μl and 200 μl) was used for background and control determination. An appropriate amount of chymotrypsin or trypsin was added to each well, except for the background determination where only buffer was added, and plates incubated for five minutes at room temperature. Typically, a single concentration of enzyme (6.25 μg per well) was used, although fractions from affinity chromatography and HPLC were assayed using one μg of enzyme per well.

After incubation, 50 μl of 5 mM N-benzoyl-L-tyrosine p-nitroanalide (BTPNA; substrate for chymotrypsin) or 4 mM N -benzoyl-DL-arginine p-nitoanalide (BAPNA; substrate for trypsin), dissolved in dimethylformamide, was added to each well and the absorbance at 415 nm read immediately using a Model 3550 microplate reader (Bio-Rad Laboratories Ltd., Hercules, California, USA). Multiple absorbance readings for each plate were recorded until the absorbance of the control (i.e. proteinase in the absence of inhibitor) reached 1.0.

Chapter 3

Results

3.1 Identification of Serine Proteinase Inhibitory activity in dry seeds of *Lolium perenne* Cultivar Grasslands Ruanui (perennial ryegrass)

The presence of serine proteinase inhibitory activity was initially observed in dry seeds of perennial ryegrass (*Lolium perenne*) cv. Grasslands Ruanui (R.W. Scott and M.T. McManus, personal communication). Thus the present project initially focused on the further characterization and purification of the serine proteinase inhibitory activity observed in these seeds.

A crude extract was obtained from milled ryegrass (*L. perenne*, cv. Ruanui) seeds. The extract was centrifuged at 20,000 g for 20 minutes at 4°C and the resultant supernatant was assayed for protein, and the presence of inhibitory activity against both chymotrypsin and trypsin. Inhibitory activity was detected against chymotrypsin but negligible inhibitory activity was observed against trypsin (data not shown). There is, however, concern over the reliability of this data because of the dark brown colour of the sample. In particular, high concentrations of extracts were used to assay for trypsin inhibitory activity resulting in a very dark colour in the assay buffer. This dark colour interfered with the inhibitor assays because it resulted in high background readings. For chymotrypsin inhibition, less extract was used and so this data can be interpreted with more confidence. Figure 3.1 shows a titration of the crude inhibitor preparation against chymotrypsin where 175 μg crude protein was required to cause 50% chymotrypsin (6.25 μg) inhibition.

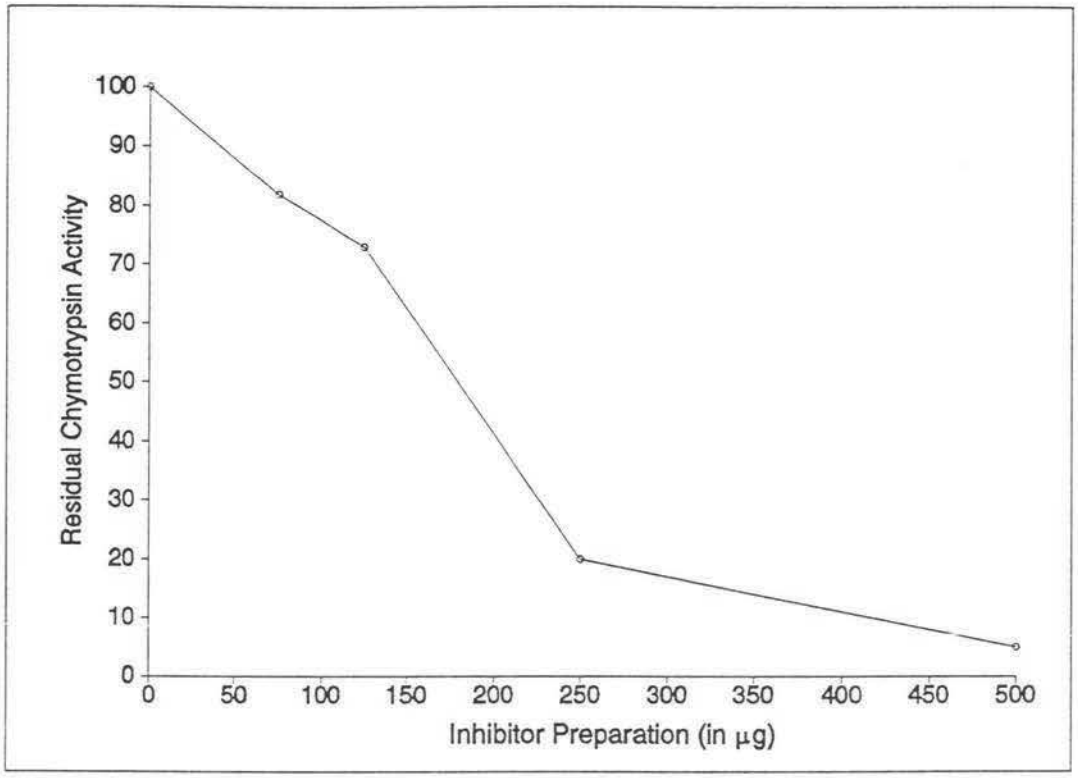
The chymotrypsin inhibitory activity was already known to be substantially stable at low pH (pH 3) and high temperature (80°C for 10 min; R.W. Scott and M.T. McManus, personal communication) and so this procedure was adopted as a first step for the purification of the inhibitor.

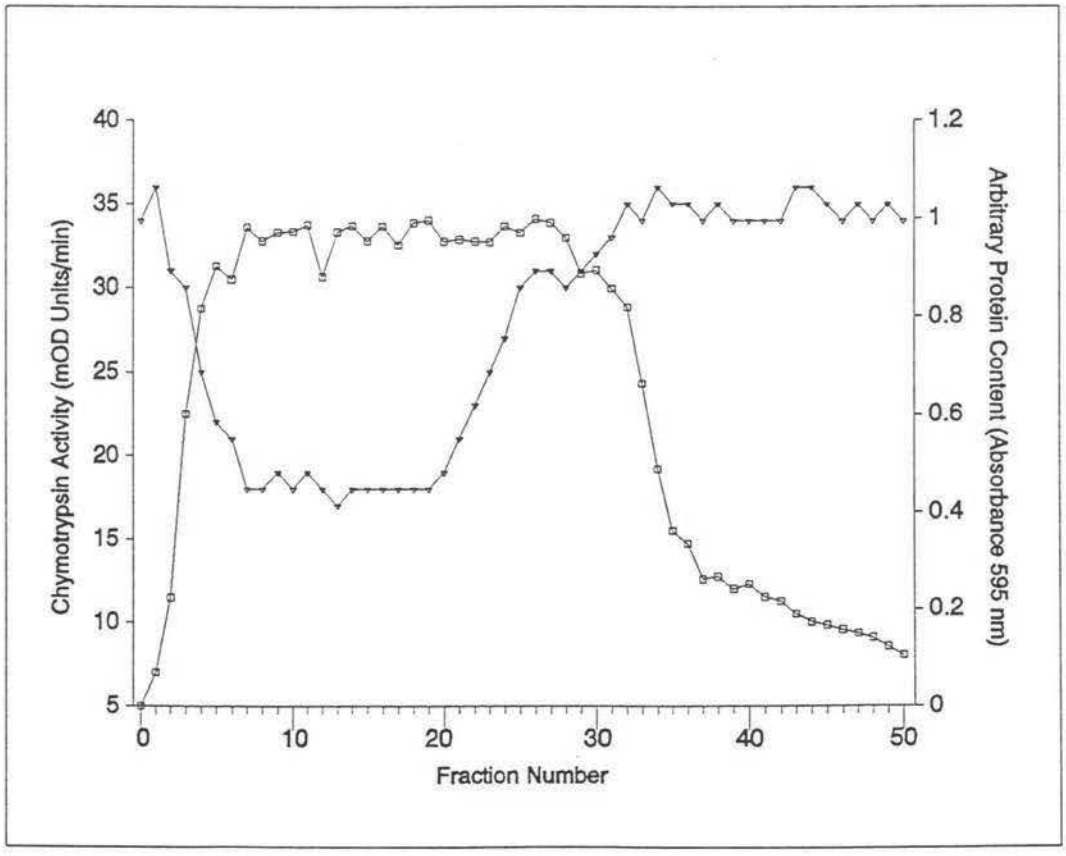
The pH of the crude extract was reduced to pH 3 with 1 N HCl, heated at 80°C for 10 minutes and then centrifuged. Sephadex G-25 gel filtration was then used to adjust the pH of the supernatant to pH 8.

Fractions from the Sephadex G-25 column were collected and assayed for the presence of protein and chymotrypsin inhibitory activity. A typical

Figure 3.1 Titration of bovine chymotrypsin with crude inhibitor protein from seeds of *Lolium perenne* cv. Grasslands Ruanui. Increasing amounts of the inhibitor preparations were added to a single concentration of 0.25 mg/ml (6.25 μg per assay) chymotrypsin, and chymotrypsin activity determined using BTpNA as substrate.

Figure 3.2 A typical Sephadex G-25 chromatograph of an acid/heat treated extract from dry seeds of *L. perenne* cv. Grasslands Ruanui. Extract was separated using 10 mM Tris-HCl, pH 8.0, as column buffer and presence of protein (□) and chymotrypsin (▽) inhibitory activity assayed for each eluate fraction.


Residual Chymotrypsin Activity
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
400
450
500
Inhibitor Preparation (in μg)


Chymotrypsin Activity (mOD Units/min)
40
35
30
25
20
15
10
5
0
10
20
30
40
50
Fraction Number
Arbitrary Protein Content (Absorbance 595 nm)
1.2
1
0.8
0.6
0.4
0.2
0

Sephadex chromatograph is given as Figure 3.2. Only one broad protein peak was eluted that contained chymotrypsin inhibitory activity. Fractions containing chymotrypsin inhibitory activity were pooled and concentrated. The concentrated G-25 sample was then re-assayed for protein and chymotrypsin inhibitory activity and was found to contain substantial activity.

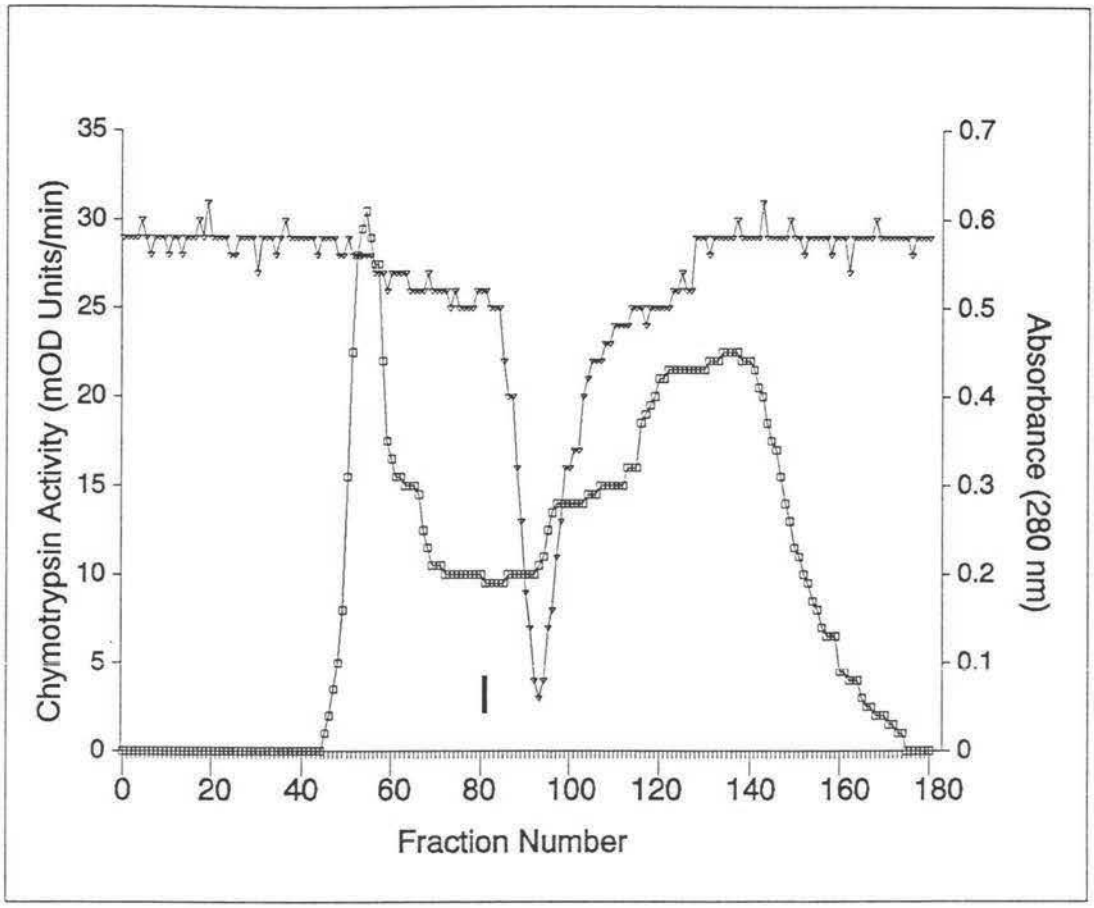
To further purify the inhibitory activity, Sephadex G-75 gel filtration was used. Gel filtration is used to separate proteins according to their molecular weights. The column was previously calibrated with standard proteins (see figure 2.1A). Fifty mg of sample was applied and fractions (2.5 ml) were collected. The absorbance of each fraction was read at 280 nm to determine protein content and each was also assayed for chymotrypsin inhibitory activity. The elution profile of Sephadex G-75 chromatography of chymotrypsin inhibitor from dry seeds of *L. perenne* cv. Grasslands Ruanui is given as Figure 3.3. Two major protein peaks were resolved. The first narrow peak (fractions 46-60) eluted as the void volume while the second broader peak (fractions 84-150) represents proteins being resolved by the column. Chymotrypsin inhibitory activity eluted at the start of second peak (fractions 87-99), and a molecular weight of *ca.* 20-22 kDa was calculated for the inhibitor.

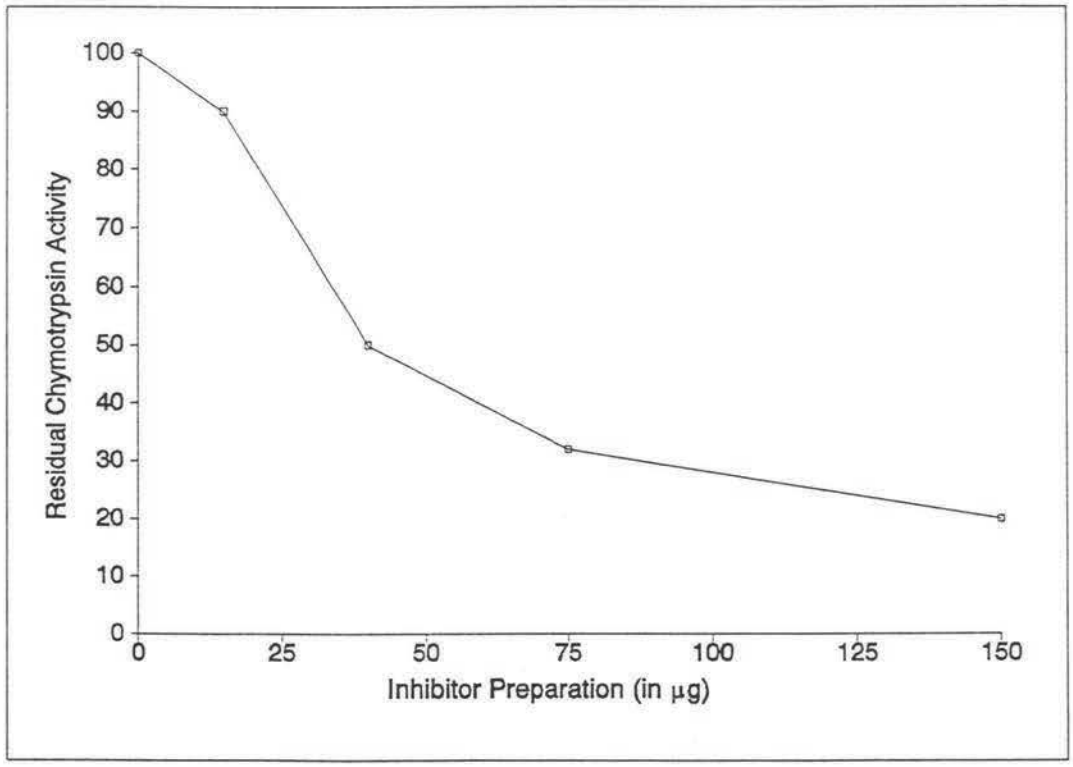
The fractions containing inhibitory activity were pooled and concentrated to a final protein concentration of 2 mg/ml. Known amounts of the inhibitor preparation were then titrated against a single amount (6.25 μg) of chymotrypsin to find 50% inhibition. As shown in Figure 3.4, 40 μg purified inhibitor inhibited chymotrypsin activity by 50%. This preparation represents a four fold purification when compared with crude inhibitor preparation (here, 175 μg of protein caused 50% chymotrypsin inhibition; Figure 3.1).

To check the purity of the pooled Sephadex G-75 fractions, samples were run through a 17.5% SDS-PAGE gel. Identical gels were stained for protein and chymotrypsin activity to visualise the inhibitory peptides. On the gel stained for proteinase activity, inhibitory activity is detected as a clear region against a pink background. Proteinase activity indicated that inhibitor activity comprised lower molecular weight peptides (*ca.* 11-12 kDa; Figure 3.5A; arrowed), the larger of which (*ca.* 12 kDa) was also identified by protein staining (Figure 3.5B; arrowed).

Figure 3.3 Chromatography of an acid/heat treated extract from dry seeds of *L. perenne* cv. Grasslands Ruanui through a 2.5 cm x 100 cm Sephadex G-75 column. The extract was separated using 100 mM Tris-HCl, pH 8.0, as column buffer and absorbance at 280 nm (□) and chymotrypsin inhibitory activity (▽) assayed for in each eluate fraction.

Figure 3.4 Titration of bovine chymotrypsin with a Sephadex G-75 purified (as shown in Figure 3.3) inhibitor protein from *L. perenne* cv. Grasslands Ruanui. Increasing amounts of inhibitor were added to a single concentration of 0.25 mg/ml (6.25 μg per assay) chymotrypsin, and chymotrypsin activity determined using BTpNA as substrate.


Chymotrypsin Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
0
5
10
15
20
25
30
35
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
20
40
60
80
100
120
140
160
180


Residual Chymotrypsin Activity
Inhibitor Preparation (in µg)
0
10
20
30
40
50
60
70
80
90
100
25
50
75
100
125
150

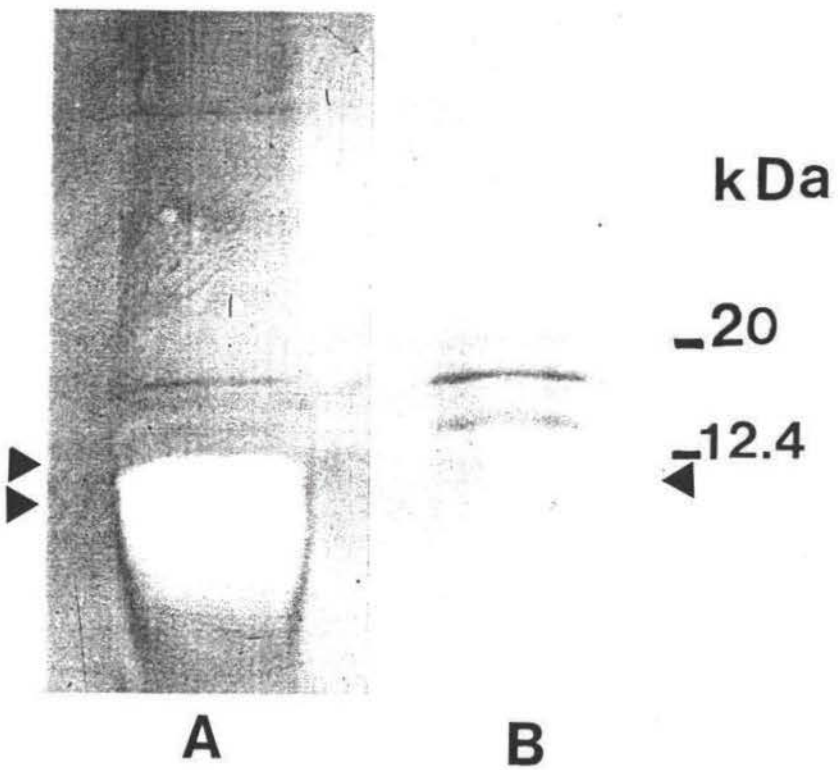


Figure 3.5 SDS-PAGE of the chymotrypsin inhibitor preparation purified from *Lolium perenne* cv. Grasslands Ruanui after gel filtration (see Figure 3.3). Proteins were separated through a 17.5% SDS-polyacrylamide gel at 25 mA constant current for 6 h, and the separated proteins stained for chymotrypsin inhibitory activity (**A**) and for protein (**B**). The MW-SDS-70L kit (Sigma) were used for standards.

On the basis of the results obtained at this stage, it was concluded that dry seeds of *L. perenne* cv. Grasslands Ruanui contain an acid/heat stable chymotrypsin inhibitor. It is most probable that similar or more potent chymotrypsin or trypsin or dual trypsin/chymotrypsin inhibitors exist in seeds of other cultivated pasture grass species. Therefore, at this stage, it was decided that before further purification, a survey of a range of seeds from pasture grass species should be conducted to see if similar or more potent chymotrypsin inhibition and trypsin inhibition exists in other species. If so, the most potent serine proteinase inhibitors can be purified further.

3.2 Survey of Serine Proteinase Inhibitory Activity in Seeds of Pasture Grass Species

Thirteen different cultivated pasture grass species were selected to study for the presence/absence of serine proteinase inhibitors. One cultivar from each species was selected and seeds were milled to fine flour.

The milled flour from dry seeds of 13 different cultivars of pasture grass species was extracted with buffer, the slurry centrifuged and the supernatant assayed for protein and the presence of inhibitory activity against bovine trypsin and chymotrypsin determined (Table 3.1). Chymotrypsin inhibition was detected in extracts of 9 out of 13 species with the extract of seeds from *Festuca arundinaceae* cv. Grasslands Roa exhibiting the most potent activity when expressed as per μg protein causing 50% chymotrypsin inhibition.

Five other cultivars of *F. arundinaceae*, were subsequently screened for bovine chymotrypsin inhibitory activity (Table 3.2). Extracts from all 5 cultivars tested inhibited chymotrypsin strongly with the most potent among those being from cv. Grasslands Garland, a turf grass.

In contrast, trypsin inhibition (50%) was negligible in all extracts tested at this level of purification, unless very high concentrations of extract were used. Trypsin inhibition was detectable in those cultivars that showed chymotrypsin inhibition but never reached 50% inhibition. However, because of the high protein concentrations needed to assay for trypsin inhibition, the

Table 3.1 Survey of trypsin and chymotrypsin inhibitory activity in extracts of seeds from various pasture grass.

			μg crude protein required for 50% inhibition of	
Species	Common name	Cultivars	Chymotrypsin	Trypsin
Lolium perenne	Perennial ryegrass	Grasslands Ruanui	148 ± 40	N.D
L. boucheanum	Hybrid ryegrass	Grasslands Greenstone	121 ± 34	N.D
L. multiflorum	Italian ryegrass	Grasslands Tama	150 ± 50	N.D
Festuca arundinacea	Tall fescue	Grasslands Roa	61 ± 22	N.D
Dactylis glomerata	Cocksfoot	Grasslands Wana	N.D	N.D
Bromus stamineus	Grazing brome	Grasslands Gala	320 ± 26	N.D
B. inermis	Smoth brome	Grasslands Tiki	N.D	N.D
B. sitchensis	Upland brome	Grasslands Hakari	N.D	N.D
B. willdenowii	Prairie brome	Grasslands Matua	> 500	N.D
Agrostis capillaris	Brown top	Grasslands Muster	N.D	N.D
Phalaris aquatica	Harding grass	Grasslands Maru	150	N.D
Phleum pratense	Timothy	Grasslands Kahu	82 ± 26	N.D
Paspalum dilatatum	Dallis grass	Grasslands Raki	300 ± 10	N.D

N.D = Not Detectable.

Table 3.2 Chymotrypsin inhibitory activity in crude extracts of different cultivars of *F. arundinaceae*

Cultivar	Accession number	μg crude protein required for 50% Chymotrypsin inhibition
Grasslands Roa	T 1347	55 ± 11
Mazark	T 1785	39 ± 3
Triumph	T 1783	53 ± 4
Grasslands G48	T 1768	40 ± 2
Grasslands Garland	T 1747	26 ± 8
S170	T 1724	49 ± 4

colour of the extract interfered with the proteinase assay, and so this data is not deemed reliable.

The objective of these experiments was to survey different seed lots for potent inhibitors of trypsin and chymotrypsin. Accordingly, the enzyme concentration used in the assays was high (6.25 μg in total volume of 275 μl). It is possible, however, that if the concentration of trypsin was lower, more trypsin inhibition could have been detected at this stage.

In order to see if the content of the chymotrypsin inhibitors in seeds was consistent or varied in response to environmental factors, the seeds of cvs. Grasslands Roa and Grasslands Ruanui from different years of harvest were extracted and the extracts were assayed for chymotrypsin inhibitory activity (Table 3.3; Table 3.4). Chymotrypsin inhibitory activity was present in extracts of all seed lots with relatively little variation in activity in most of the seed lines from year to year, or between different harvests from the same year. However, two lines from cv. Grasslands Ruanui demonstrated a higher chymotrypsin inhibitor content. One of these two (A4729) had about a two fold increase in inhibitor content, while the second one (A4345) had one third increase. However, only seeds of A4798 were available in sufficient amount for a larger extraction, and so this line was used for further experiments.

3.3 Preliminary Purification of Proteinase Inhibitory Activity

From the initial screen, the two cultivars of *F. arundinaceae*, cv. Grasslands Roa and cv. Grasslands Garland, that demonstrated particularly potent chymotrypsin inhibition were selected for further investigation. As a comparison, two cultivars of the economically important grass genus *Lolium* was selected. The first was the cultivar that had already been investigated, *L. perenne* cv. Grasslands Ruanui while the second *L x boucheanum* cv. Grasslands Greenstone also demonstrated relatively potent inhibition.

Table 3.3 Chymotrypsin inhibitory activity in crude extracts of *F. arundinacea* (cv. Grasslands Roa) seed representing different years of harvest.

Accession Number	Year of harvest	μg crude protein caused 50% chymotrypsin inhibition
T1766	1991	33 ± 3
T1765	1991	32 ± 4
T1661	1989	30 ± 4
T1659	1988	30 ± 2
T1607	1988	26.25
T1606	1987	38 ± 6
T1347⊗	1986	51 ± 7
T1329	1985	36 ± 4

Table 3.4 Chymotrypsin inhibitory activity in crude extracts of *L. perenne* (cv. Grasslands Ruanui) seed representing different years of harvest.

Accession Number	Year of harvest	μg crude protein caused 50% chymotrypsin inhibition
A3731	January 1978	140 ± 16
A3799	February 1979	151 ± 14
A3840	January 1980	157 ± 15
A3847	January 1981	151 ± 14
A4075	January 1982	121 ± 16
A4345	June 1983	108
A4354	June 1983	115 ± 11
A4659	August 1984	121 ± 16
A4729	May 1985	73 ± 8
A4798⊗	May 1986	121 ± 16
A4807	July 1986	121 ± 10

⊗ Line used in survey

3.3.1 Stability at low pH and high temperature

The stability of chymotrypsin inhibitory activity (of the four cultivars selected) was investigated at low pH (pH 3) and high temperature (80°C). Table 3.5 shows that more than 50% inhibitory activity was recovered after acid/heat denaturing and G-25 chromatography from the two *Lolium* cultivars. However, the recovery from the two *Festuca* cultivars was 39%. Because most serine proteinase inhibitors reported so far are acid and heat stable, we continued with the purification of the acid/heat stable inhibitors. The purification table indicated that there was still a substantial amount of chymotrypsin inhibitory activity left after acid/heat denaturing and the specific activity of the preparation increased. The lost inhibitory activity could either be due to chymotrypsin inhibitors that were partially degraded, or a completely different acid/heat labile chymotrypsin inhibitor protein that is wholly denatured during acid and heat treatment. Therefore it was decided to further purify the existing acid/heat stable inhibitors from all the four cultivars using acid/heat denaturing, Sephadex G-25 and Sephadex G-75 gel chromatography.

3.3.2 Initial Purification

For the initial purification of inhibitors, a large extract (500 ml) was obtained from each of the four cultivars selected. The extract was assayed for protein concentration and chymotrypsin inhibition.

The extract from each cultivar was subjected to low pH (3.0) and then high temperature (80°C) to denature other proteins and so select for acid and heat stable inhibitors. Precipitated proteins were collected by centrifugation and Sephadex G-25 gel filtration chromatography was used to adjust the pH of the supernatant to pH 8.0. The pattern of protein separation revealed for all four cultivars was similar and also similar to that obtained for *L. perenne* cv. Grasslands Ruanui previously (Figure 3.2). One broad protein peak was eluted that contained chymotrypsin inhibitory activity. At least 2 separate chromatography runs were performed for each cultivar. Chymotrypsin inhibitor containing fractions from different runs of each cultivar were pooled and

Table 3.5 Purification table of four cultivars for stability at low pH and high temprature

Cultivar (Grasslands)	Total volume (ml)	Total protein (mg)	Total inhibitory activity (Units)	Specific activity (Units/mg)	Purification (Fold)	Recovery (%)
Ruanui						
Crude extract	12	36	783	22	1	100
Acid-heat G-25	12	7.5	455	61	2.8	58
Greenstone						
Crude extract	12	42	1500	36	1	100
Acid-heat G-25	12	9.6	741	77	2.1	49
Roa						
Crude extract	12	45	3309	73	1	100
Acid-heat G-25	12	8.4	1280	152	2.1	39
Garland						
Crude extract	12	60	6579	110	1	100
Acid-heat G-25	12	10.8	2177	202	2	38.5

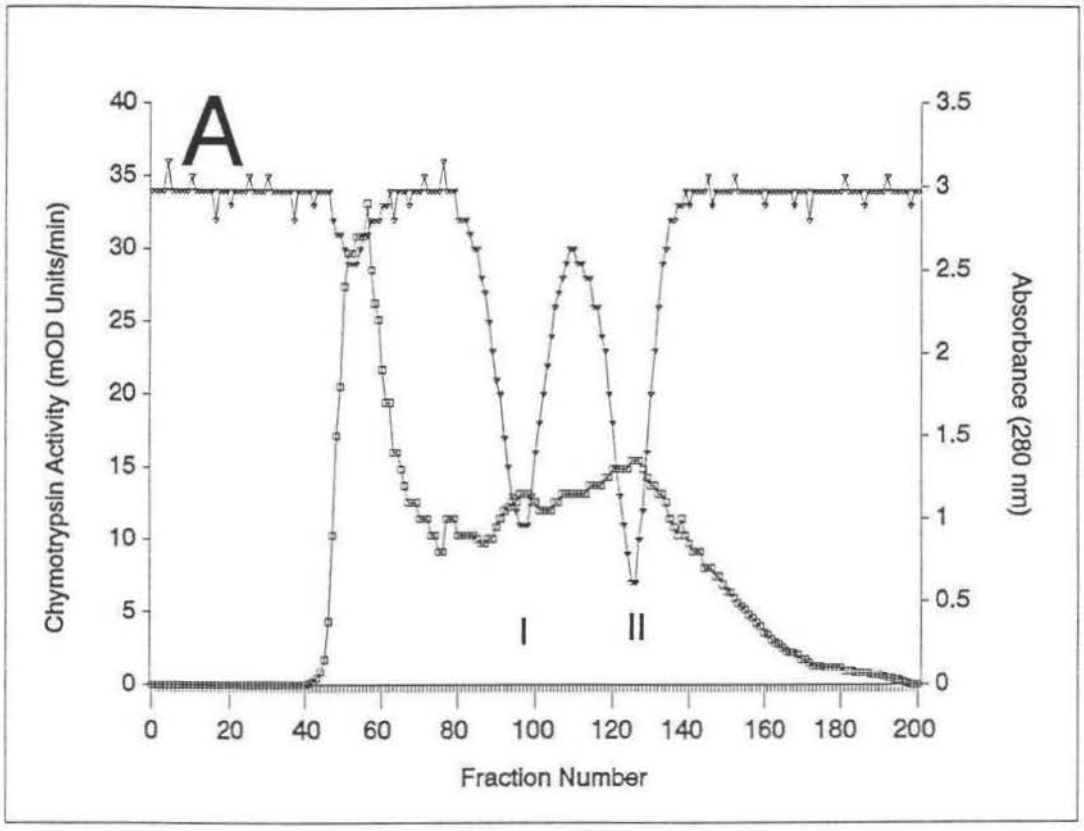
concentrated. The concentrate obtained was cleared by centrifugation, assayed for protein concentration and chymotrypsin inhibition.

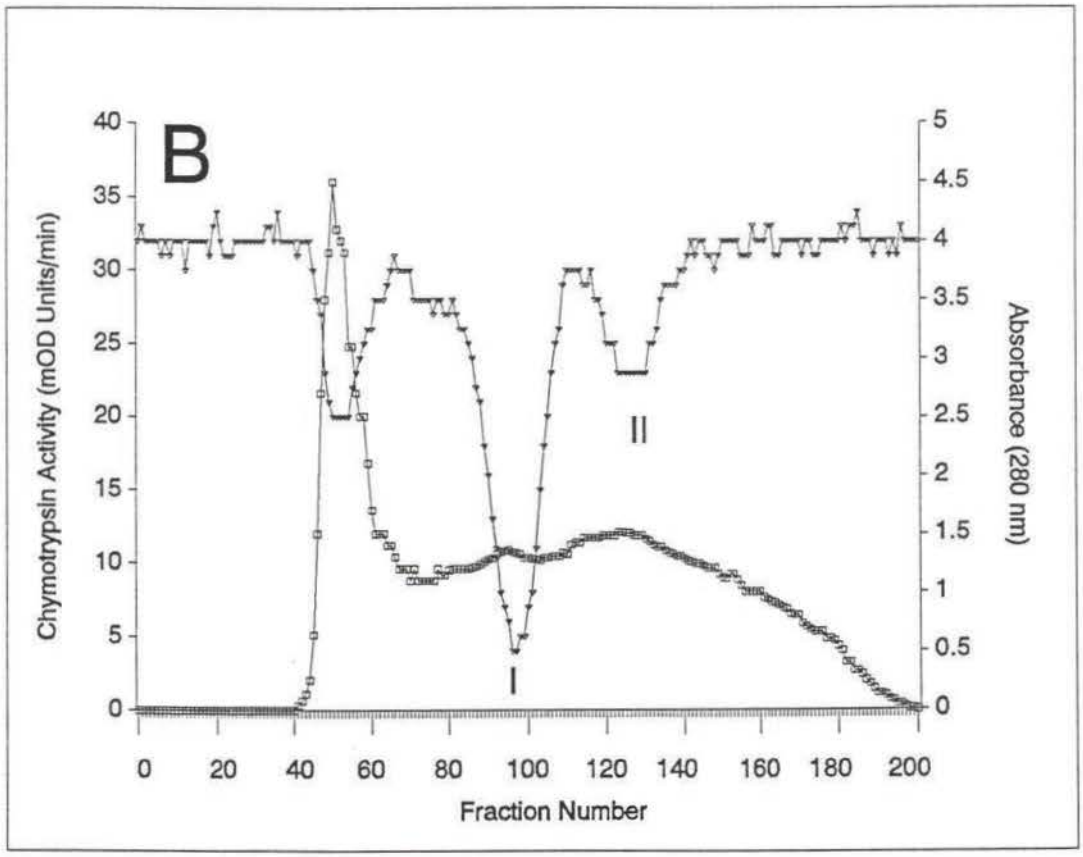
3.3.3 Partial purification

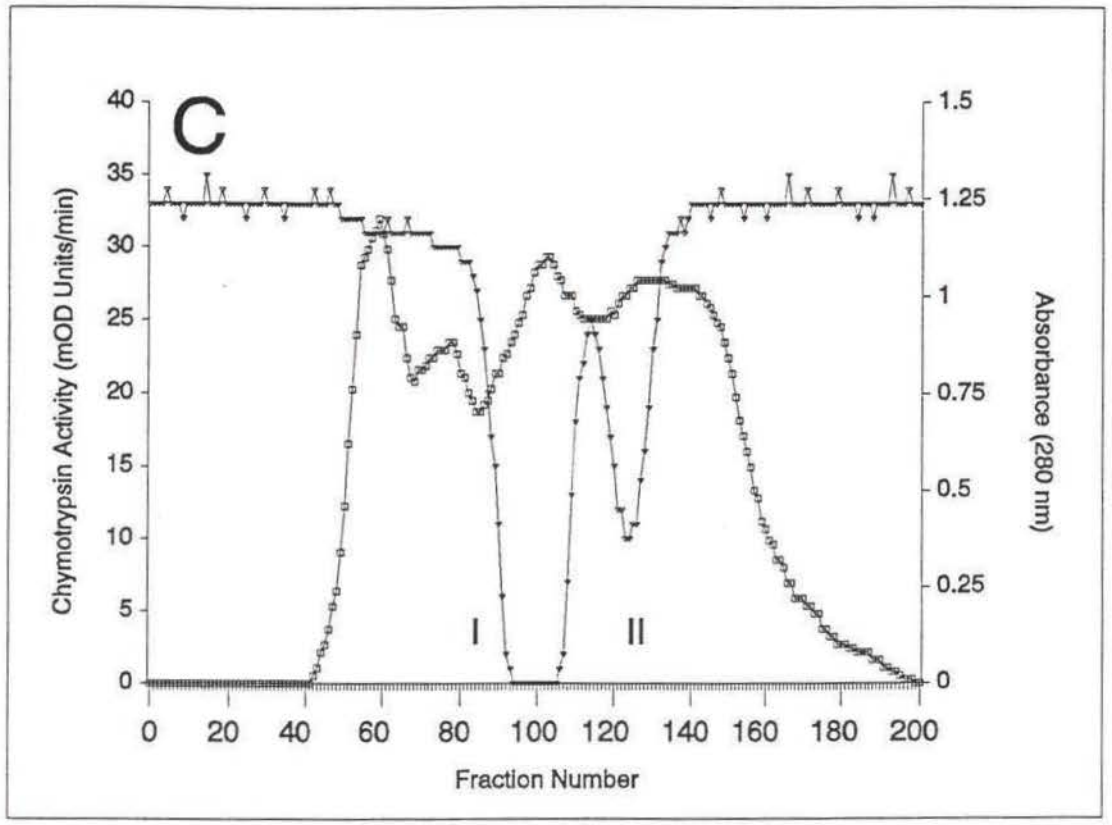
Concentrated Sephadex G-25 fractions from all the four cultivars were used for Sephadex G-75 gel chromatography to purify the inhibitor further. Fractions (2.5 ml) were collected and assayed for protein and chymotrypsin inhibition. The elution profile of proteins and inhibitory activity from each cultivar after Sephadex G-75 chromatography is given as Figures 3.6A-D. Two different chymotrypsin inhibitory peaks were observed for each cultivar, and designated as peak I and peak II. In each cultivar, peak I had a molecular weight of *ca.* 20-22 kDa, while peak II had a molecular weight of *ca.* 10-12 kDa. Peak I in the two *Festuca* cultivars (cvs. Grasslands Garland and Grasslands Roa) was broader than peak I from two *Lolium* cultivars (cvs. Grasslands Ruanui and Grasslands Greenstone), suggesting that the inhibitor from the two *Festuca* cultivars is either more potent or present in a higher concentration. Peak II from *Festuca* cultivar Grasslands Garland was broader than peak II from all the other cultivars. Peak II from *Festuca* cultivar Grasslands Roa and *Lolium* cultivar Grasslands Ruanui was similar. A very small peak II was observed from Grasslands Greenstone.

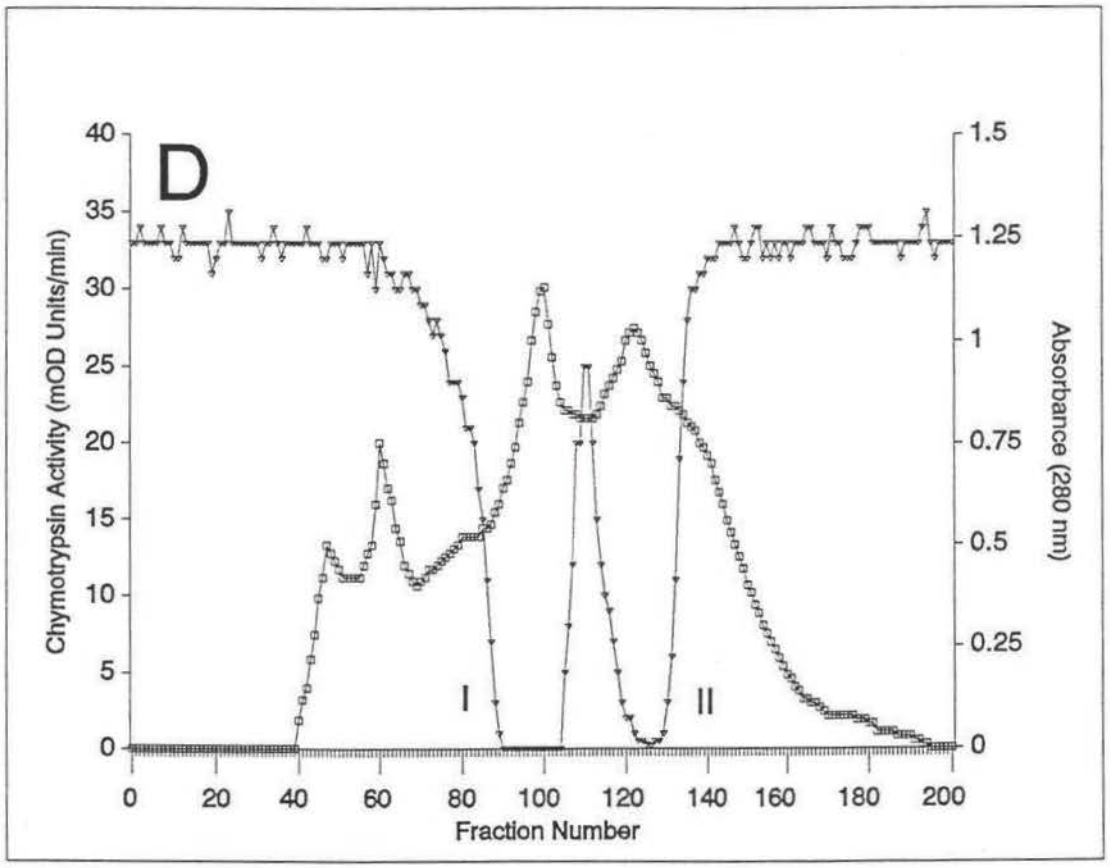
The partially purified inhibitory peaks from all four cultivars were concentrated separately. Protein and inhibitor assays of the two peaks from all the four cultivars indicated that most protein eluted in peak I while peak II had only a fraction of peak I in terms of protein. Peak I, therefore, was chosen for further experiments. To compare the potency of inhibitory activity in each of the four cultivars, an increasing amount of peak I material was titrated against a single concentration of chymotrypsin (6.25 μg; Figure 3.7). The titration result shows that the inhibitor from the two *Festuca* cultivars, particularly cv. Grasslands Roa, were more potent inhibitors of chymotrypsin when compared with the two *Lolium* cultivars. For example, 20 μg of the inhibitor preparation from *F. arundinaceae*, cv. Grasslands Roa was sufficient to inhibit the enzyme by 50%, while 45 μg of the inhibitor preparation from *L. perenne,* cv.

Figure 3.6 Chromatography through a 2.5 cm x 100 cm Sephadex G-75 column of acid/heat treated extracts from dry seeds of *Lolium perenne* cv. Grasslands Ruanui (**A**), *L. x boucheanum* cv. Grasslands Greenstone (**B**), *Festuca arundinaceae* cv. Grasslands Roa (**C**), *F. arundinaceae* cv. Grasslands Garland (**D**). Extracts were separated using 100 mM Tris-HCl, pH 8.0, as column buffer and absorbance at 280 nm (□), and chymotrypsin inhibitory activity (▽) assayed for in each eluate fraction. Two peaks of inhibitory activity were present and designated as peak I and II.


A
Chymotrypsin Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
I
II
0
5
10
15
20
25
30
35
40
0
0.5
1
1.5
2
2.5
3
3.5
0
20
40
60
80
100
120
140
160
180
200


B
Chymotrypsin Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
I
II
0
5
10
15
20
25
30
35
40
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
20
40
60
80
100
120
140
160
180
200


C
Chymotrypsin Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
I
II
0
5
10
15
20
25
30
35
40
0.25
0.5
0.75
1
1.25
1.5
0
20
40
60
80
100
120
140
160
180
200


D
Chymotrypsin Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
I
II
0
5
10
15
20
25
30
35
40
0.25
0.5
0.75
1
1.25
1.5
0
20
40
60
80
100
120
140
160
180
200

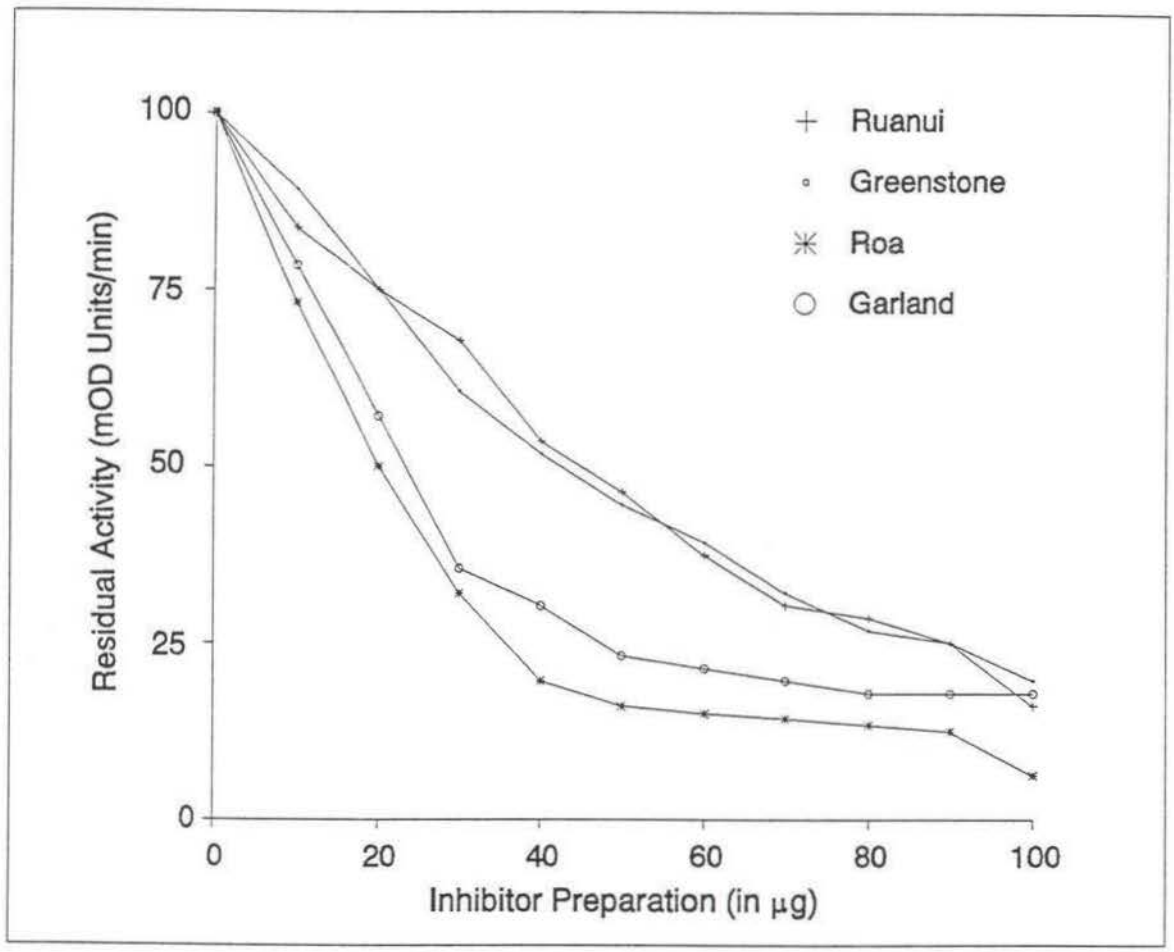


Figure 3.7 Titration of bovine chymotrypsin with peak I preparations from Sephadex G-75 purified inhibitor proteins (as shown in Figure 3.6A-D) from two *Festuca* cultivars and two *Lolium* cultivars. Increasing amounts of inhibitor were added to a single concentration of 0.25 mg/ml (6.25 μg per assay) chymotrypsin, and chymotrypsin activity determined using BTpNA as substrate.

Grasslands Ruanui was needed to obtain the same level of inhibition. Therefore, it was decided to further purify the inhibitor from one of the *Festuca arundinaceae* cultivars, cv. Grasslands Roa.

Peak I from *F. arundinaceae* cultivar Grasslands Roa was separated through a 17.5% SDS-PAGE gel, and stained for chymotrypsin inhibitory activity (Figure 3.8A) and for protein (Figure 3.8B). At least 6 low molecular protein bands (Figure 3.8B; lane a; arrowed) were present that corresponded to chymotrypsin inhibitory region on the activity gel (Figure 3.8A; lane a; arrowed), ranging in molecular weights from *ca.* 3 kDa to 12 kDa. Comparison of gel electrophoresis of peak I from the two genera indicates that the *Lolium* cultivar contained only two inhibitor bands (Figures 3.5A,B) while the *Festuca* cultivar contained 6 inhibitor bands (Figures 3.8A,B). However, the two inhibitor bands (11 kDa and 12 kDa) present in *Lolium* (Figurse 3.5A,B) were also present in *Festuca* (Figures 3.8A,B), but the lower molecular bands were absent in *Lolium* (Figures 3.5A,B).

3.3.4 Ion exchange chromatography of the chymotrypsin inhibitor from *F. arundinaceae* cultivar Grasslands Garland

Initially, an inhibitor preparation (peak 1) from cv. Grasslands Roa was applied to a small (5 ml) column of either Q-Sepharose (anionic exchange resin column; column buffer was 25 mM Tris-HCL pH 7.5), or S-Sepharose (cationic exchange resin column; column buffer was 25 mM sodium acetate pH 4.5). For S-Sepharose, the inhibitor was dialysed against sodium acetate (column buffer) overnight. However, for Q-Sepharose, as the inhibitor was already in 100 mM Tris-HCl pH 8 buffer, no dialysis was necessary. The inhibitor protein did not bind to either of the two ion exchange columns and eluted with unbound proteins (data not shown). For this reason, the further purification of the chymotrypsin inhibitors in cv. Grasslands Roa was discontinued. Instead, the inhibitory activity in peak I of cv. Grasslands Garland was resolved using ion-exchange chromatography.

When applied to Q-Sepharose this inhibitory activity eluted with the unbound proteins (data not shown). However, when the inhibitor preparation was applied to S-Sepharose, inhibitory activity eluted as a single peak within

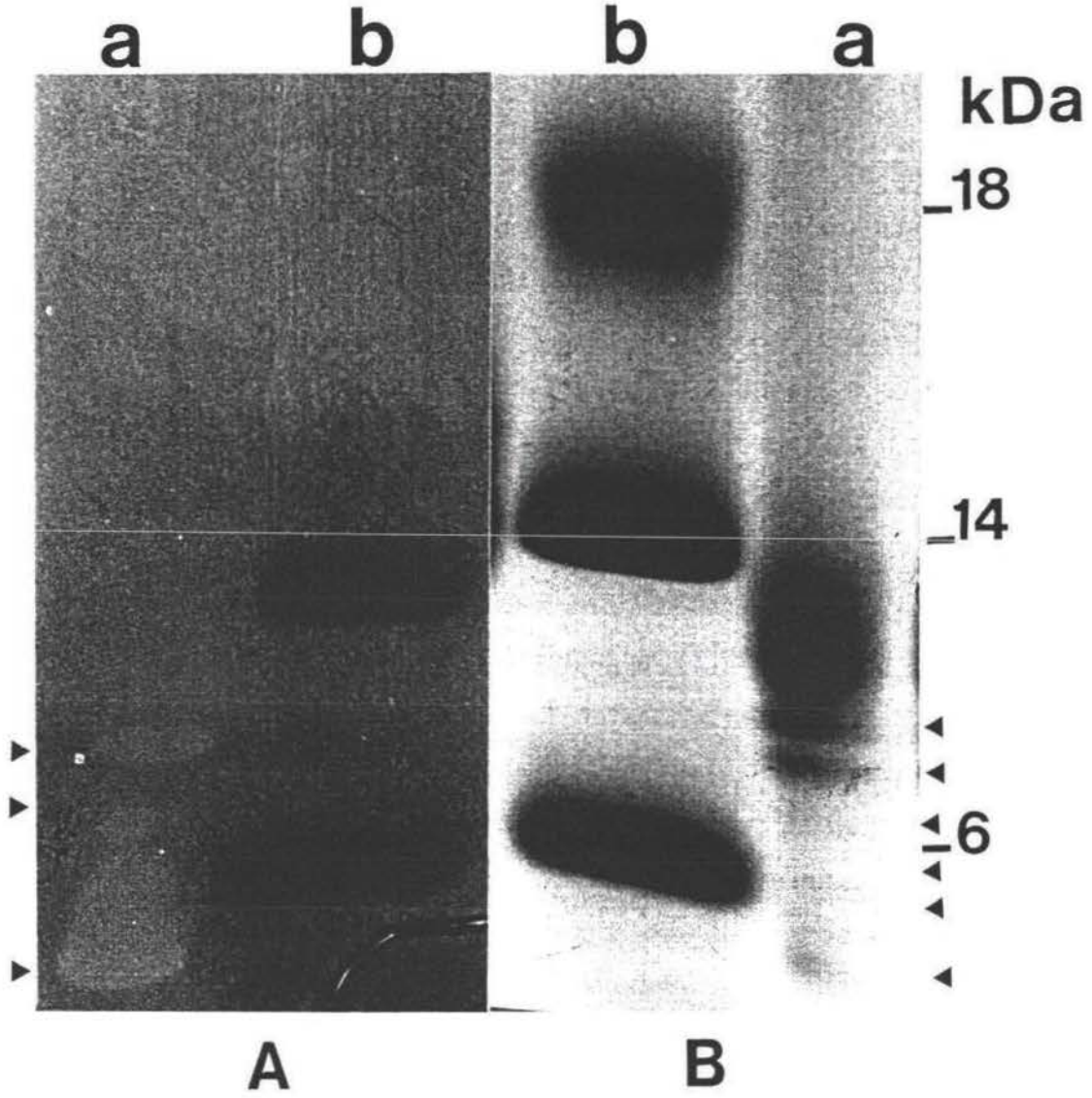


Figure 3.8 SDS-PAGE of the chymotrypsin inhibitors from *F. arundinaceae* cv. Grasslands Roa purified as peak I using Sephadex G-75 (see Figure 3.6C). Proteins were separated through a 17.5% SDS-polyacrylamide gel at 25 mA constant current for 6 h, and the separated proteins stained for chymotrypsin inhibitory activity (**A**) and for protein (**B**). Lane **a** Sephadex G-75 peak I; **b**; Standards. Prestained protein molecular weight standards, low range (GIBCO BRL, Life Technology, Gaithersburg, USA) were used.

a salt gradient ranging from 0 to 0.5 KCl (Figure 3.9). This result indicated that chymotrypsin inhibitory activity from *F. arundinaceae* cv. Grasslands Garland peak I is positively charged at pH 4.5 (25 mM sodium acetate). Fractions containing inhibitory activity were pooled and concentrated and dialysed against 50 mM Tris-HCl pH 8.0.

Using gel electrophoresis (17.5% SDS-PAGE), the inhibitor was found to contain two major inhibitor bands after protein staining with molecular weights of *ca.* 11 kDa and 12 kDa (Figure 3.10A; arrowed). These also showed activity against chymotrypsin after activity staining (Figure 3.10B; lane a). To ensure that there was no loss of inhibitor proteins after the dialysis step that was used to prepare sample for ion exchange, the inhibitor preparations after Sephadex G-75 chromatography, before and after dialysis were also separated, and the gel was stained for chymotrypsin inhibitory activity (Figure 3.10B; lanes c,d). There was no difference between these two samples. The activity pattern for these two samples was also similar to the inhibitor sample after ion exchange indicating that the lower molecular weight inhibitory peptides are related to the higher molecular weight bands (Figure 3.10B). However, these lower molecular peptides could not be visualised in the protein gel (Figure 3.10A) probably because these were present in very small concentrations. The two protein bands that were observed after ion exchange chromatography (Figure 3.10A; arrowed), were transferred to PVDF membrane and 12 amino acids of the N-terminal sequenced (see next section).

3.3.5 N-terminal amino acid sequence of the chymotrypsin inhibitor purified by ion exchange chromatography

The two inhibitor bands that were transferred to PVDF membrane were sequenced by Dr David Christie, Department of Biochemistry, Auckland University, Auckland, New Zealand. The first 12 N-terminal amino acids were determined for each protein band. The 12 kDa inhibitor band was sequenced with confidence (Figure 3.11A), while the 11 kDa inhibitor band appeared to contain a number of sequences and so was probably not pure enough for sequencing. For comparative purposes, an alignment of the 12 amino acids

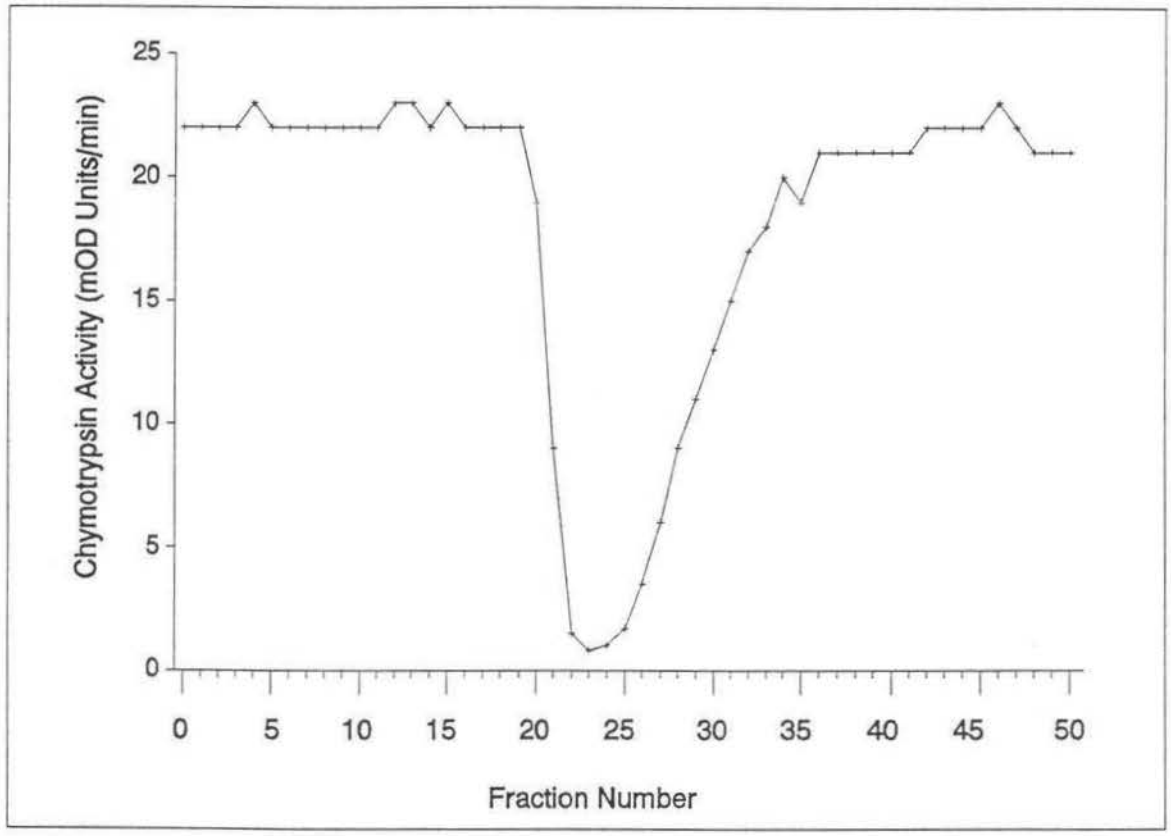


Figure 3.9 Elution profile of chymotrypsin inhibitory activity from the peak I Sephadex G-75 preparation from *F. arundinaceae* cv. Grasslands Garland (see Figure 3.6D) using ion exchange (S-Sepharose) chromatography. The sample was applied to a 5 ml S-Sepharose column previously equilibrated with 25 mM sodium acetate pH 4.5 and bound inhibitor protein eluted with a linear gradient (0.0 to 0.5 M) of KCl.

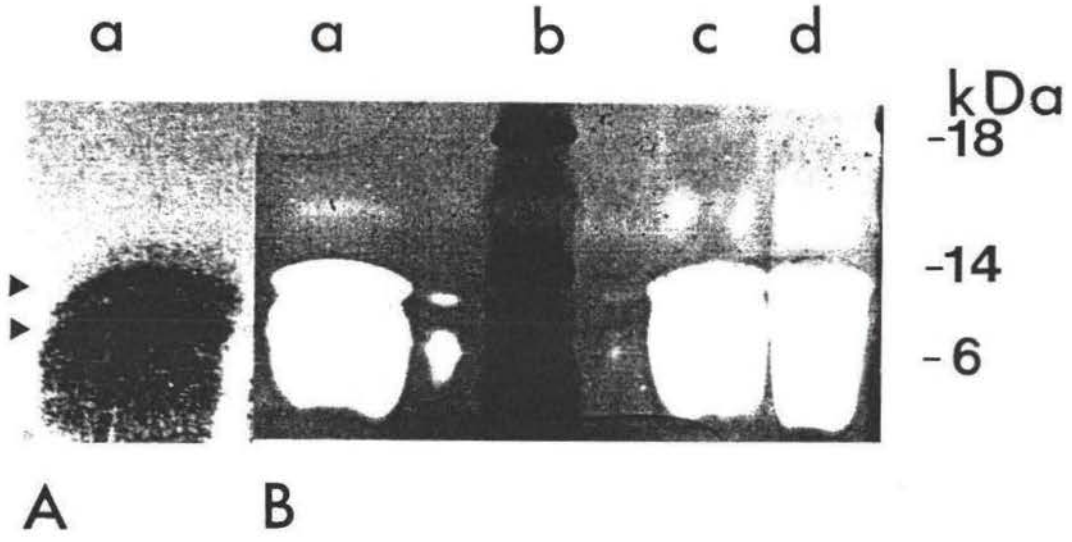


Figure 3.10 SDS-PAGE of chymotrypsin inhibitory activity purified from *F. arundinaceae* cv. Grasslands Garland by ion exchange chromatography (as shown in Figure 3.9). Proteins were separated through a 17.5% polyacrylamide gel at 25 mA constant current for 6 h, and the separated proteins stained for protein (**A**), and for chymotrypsin inhibitory activity (**B**); lane **a**, after ion exchange; **b**, standards; **c**, Sephadex G-75 peak I before dialysis; **d**, Sephadex G-75 peak I after dialysis. Prestained protein molecular weight standards, low range (GIBCO BRL, Life Technology, Gaithersburg, USA) were used.

A.

T G D Y F Y A G M G L P

B.

T G D Y F Y A G M G L P
• • • • • • • • • •
T G Q Y C Y A G M G L P

C.

T G D Y F Y A G M G L P
• • • • • • • • • •
T G P Y C Y A G M G L P

E.

T G D Y F Y A G M G L P
• • • • • • • • •
T G P Y C Y P G M G L P

Figure 3.11. A. The N-terminal sequence of the 12 kDa chymotrypsin inhibitory peptide purified from *F. arundinaceae* cv. Grasslands Garland by ion exchange (as shown in Figure 3.7). **B.** Comparison of the N-terminal sequence from the 12 kDa inhibitory peptide purified here with the cDNA deduced N-terminal sequence from insect **α**-amylase inhibitor (CMa) from barley (*H. vulgare*) seeds (Rasmussen and Johansson, 1992). **C.** Comparison with the cDNA deduced N-terminal sequence of insect **α**-amylase inhibitor (CM1) from wheat (*T. aestivum*) seeds (Garcia-Maroto *et al.*, 1990). **D.** Comparison with cDNA deduced N-terminal sequence from an **α**-amylase inhibitor, CM2 from wheat (*T. durum*) seeds (Gautier *et al.*, 1991).

sequenced from 12 kDa inhibitor only with the sequence of other known proteins was obtained by Dr. Nick Ellison, AgResearch Grasslands, Palmerston North, New Zealand using programme "Fast-A" (Pearson and Lipman, 1988). Three proteins that had the highest consensus homology were identified and are given as Figures 3.11B,C,D. The highest homology (83.3%) was found with two insect **α**-amylase inhibitors, CMa from barley (*H. vulgare*) seeds (Rasmussen and Johansson, 1992; Figure 3.11B) and CM1 from wheat (*T. aestivum*) seeds (Garcia-Maroto *et al.*, 1990; Figure 3.11C). The next best homology found was with an **α**-amylase inhibitor, CM2 from wheat (*T. durum*) seeds (Gautier *et al.*, 1991; Figure 3.11D). However, all of these three are active against **α**-amylases and therefore belong to the cereal **α**-amylase inhibitor family (Garcia-Olmedo *et al.*, 1987).

There are no reports in the literature that indicate the presence of chymotrypsin inhibitory activity in any of the three **α**-amylase inhibitors that were found to contain homology with the chymotrypsin inhibitor purified here. Indeed, only two of these **α**-amylase inhibitors, CM1 from wheat (Barber *et al.*, 1986a) and CMa from barley (Barber *et al.*, 1986b) have been assayed for inhibitory activity against trypsin. These proteins did not have inhibitory activity against trypsin, while trypsin inhibitory activity has not been assayed for CM2 from *T. durum*. The *ca.* 12 kDa protein, therefore, is probably an **α**-amylase inhibitor that exists in cv. Grasslands Garland seeds or is a storage protein that either is an **α**-amylase inhibitor or has homology with **α**-amylase inhibitors. If so, this protein co-purified (during gel filtration and ion exchange chromatography as well as SDS-PAGE) with the chymotrypsin inhibitory activity. Alternatively, the **α**-amylase inhibitor purified here could have chymotrypsin inhibitory activity. To determine the specificity of the protein more accurately and to sequence the *ca.* 11 kDa peptide, it was decided to purify these proteins to homogeneity.

3.4 Purification of a Proteinase Inhibitor to Homogeneity

To improve the resolution and to obtain a larger amount of Sephadex G-75 purified material for subsequent analysis in a single chromatography run, a larger (10 x 100 cm) column was used. For the initial purification of inhibitors,

a large extract (1500 ml) was obtained and at this stage of the extraction all four cultivars were used. Inhibitor samples for Sephadex G-75 chromatography were prepared using acid/heat denaturing and Sephadex G-25 chromatography in a similar way to that described previously.

3.4.1 Partial purification of proteinase inhibitors

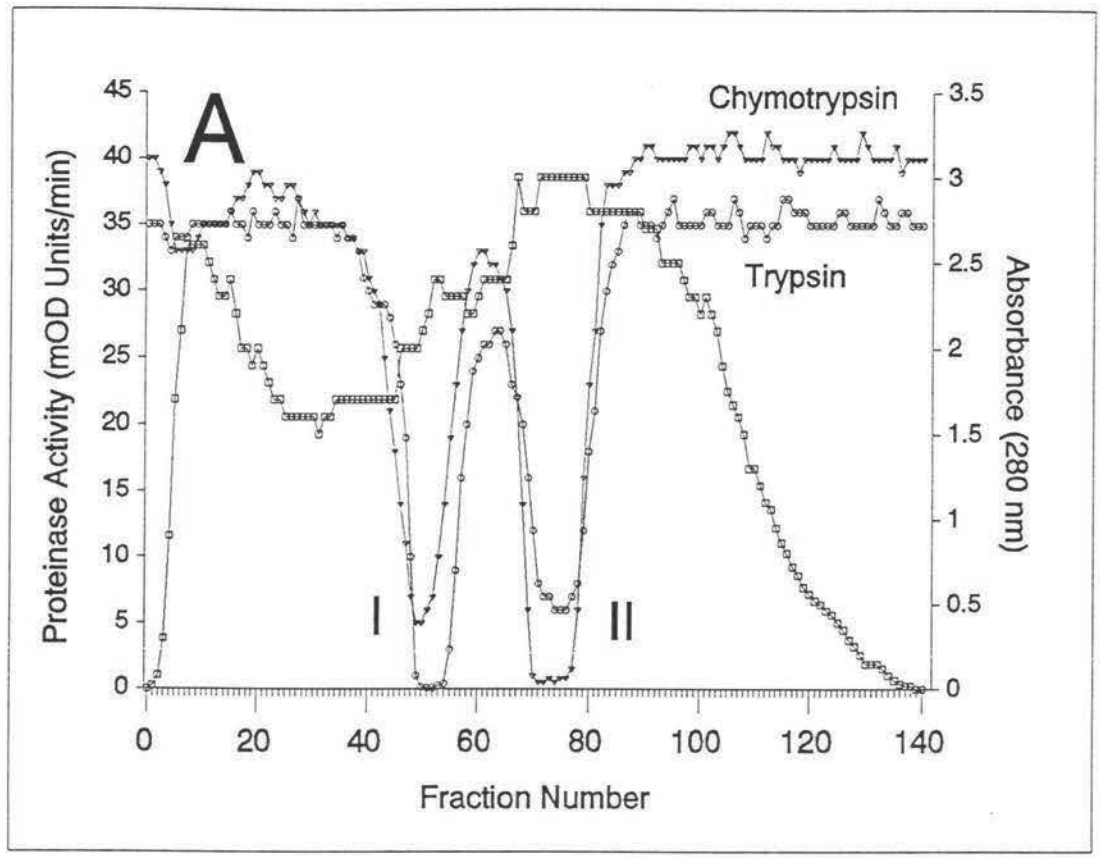
Sephadex G-75 gel filtration chromatography was used for the partial purification of the inhibitors from each cultivar. Concentrated samples were applied to a Sephadex G-75 column and fractions (12 ml) were collected after the void volume had been discarded. The absorbance at 280 nm of each fraction was used to determine protein content. Each fraction was also assayed for inhibitory activity against bovine trypsin and chymotrypsin.

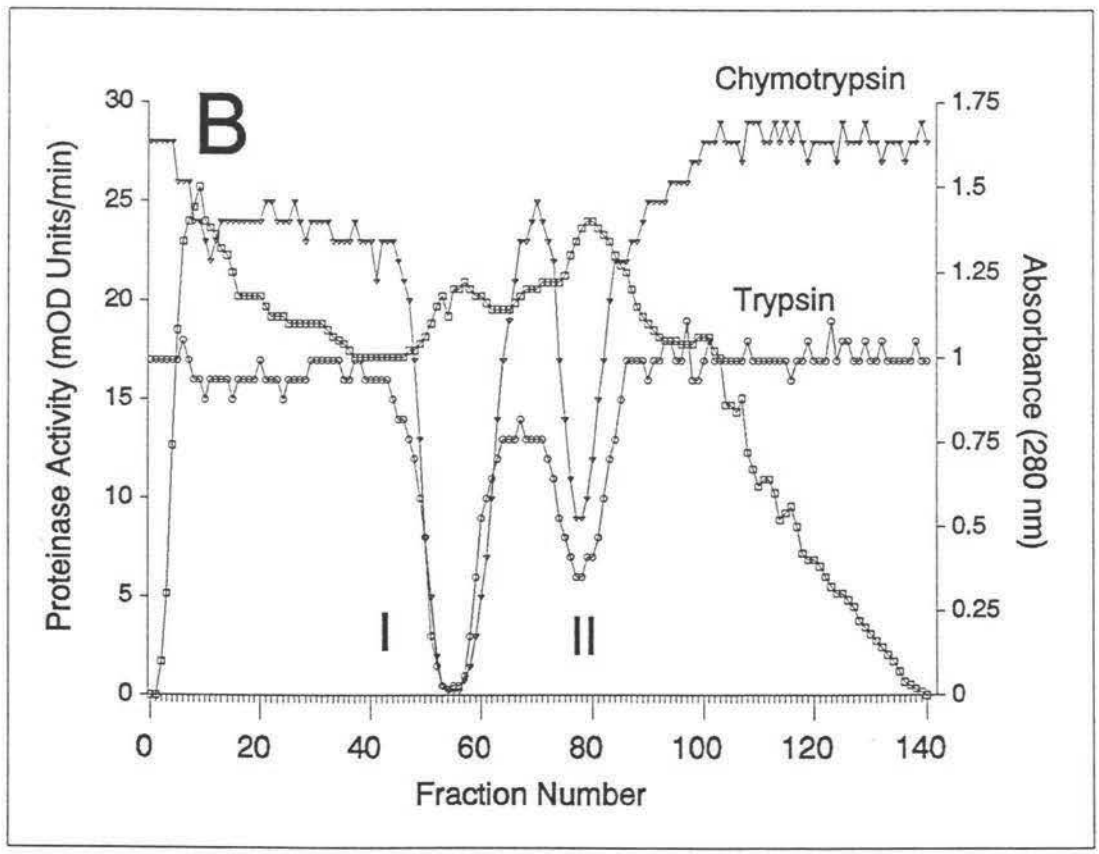
Figures 3.12A-D shows the elution profile of proteins and inhibitory activity from each of the four cultivars after large Sephadex G-75 column. In agreement with the previous Sephadex G-75 separation (see 3.3.3), the chymotrypsin inhibitory activity resolved into two distinct peaks of activity that corresponded to native molecular weights of *ca.* 20-22 kDa (designated peak I), and of *ca.* 10-12 kDa (peak II).

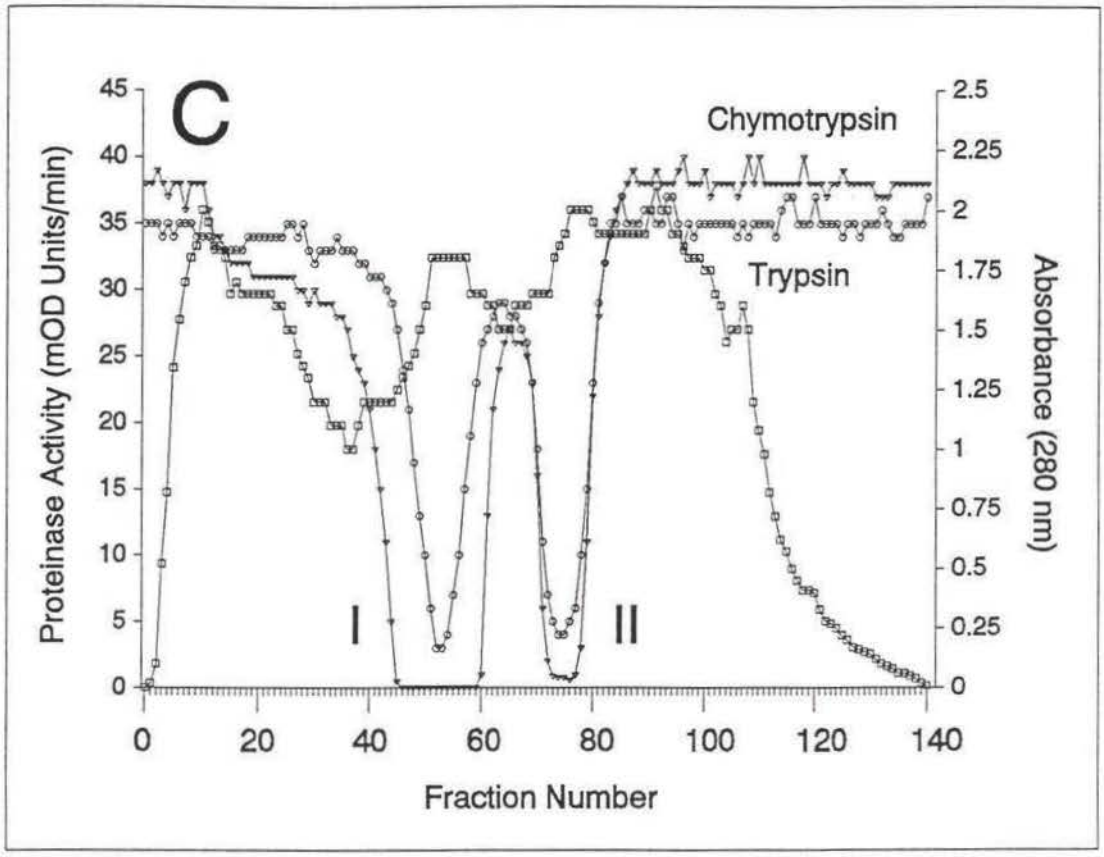
This time, trypsin inhibitory activity was also assayed in each gel filtration fraction of each cultivar and found to co-elute with chymotrypsin inhibitory activity in both peak I and II indicating similar molecular weights for each peak (Figures 3.12A-D). Fractions comprising both peaks were pooled separately and concentrated from all the four cultivars. Both peaks contained about 4 times more protein than that was obtained from the smaller G-75 column (data not shown).

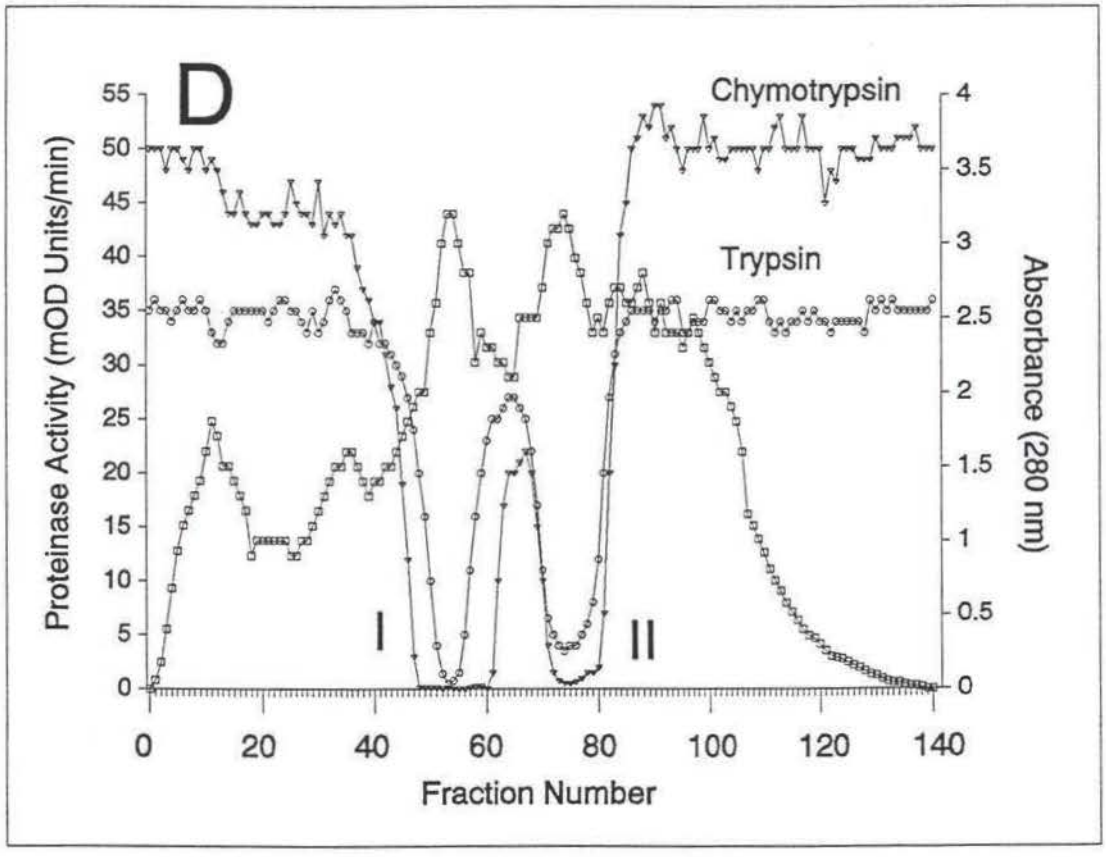
All samples (two peaks, peak I and II for four cultivars = 8 samples) were separated by gel electrophoresis (10-20% SDS-PAGE gradient gel) to ascertain the purity of each sample. Identical gels were stained for protein (Figure 3.13C) and proteinase activity staining (Figures 3.13A,B). When stained for chymotrypsin activity, different banding patterns were observed within the two gel filtration peaks (Figure 3.13A). In peak I, several different iso-inhibitor bands were present that had molecular weights ranging from *ca.* 3 kDa to 8 kDa. This

Figure 3.12 Chromatography through a 10 cm x 100 cm Sephadex G-75 column of acid/heat treated extracts from dry seeds of *Lolium perenne* cv. Grasslands Ruanui (**A**), hybrid *Lolium x boucheanum* cv. Grasslands Greenstone (**B**), *Festuca arundinaceae* cv. Grasslands Roa (**C**), *Festuca arundinaceae* cv. Grasslands Garland (**D**). Extracts were separated using 25 mM ammonium bicarbonate, as column buffer and absorbance at 280 nm (□) and chymotrypsin inhibitory activity (▽) and trypsin inhibitory activity (○) assayed for in each eluate fraction. Two peaks of inhibitory activity were present and designated as peak I and II.


A
Chymotrypsin
Trypsin
I
II
Proteinase Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
0
5
10
15
20
25
30
35
40
45
0
0.5
1
1.5
2
2.5
3
3.5
0
20
40
60
80
100
120
140


B
Chymotrypsin
Trypsin
I
II
Proteinase Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
0
5
10
15
20
25
30
0
0.25
0.5
0.75
1
1.25
1.5
1.75
0
20
40
60
80
100
120
140


C
Chymotrypsin
Trypsin
I
II
Proteinase Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
0
5
10
15
20
25
30
35
40
45
0.25
0.5
0.75
1
1.25
1.5
1.75
2
2.25
2.5
0
20
40
60
80
100
120
140


D
Chymotrypsin
Trypsin
I
II
Proteinase Activity (mOD Units/min)
Absorbance (280 nm)
Fraction Number
0
5
10
15
20
25
30
35
40
45
50
55
0.5
1
1.5
2
2.5
3
3.5
4
0
20
40
60
80
100
120
140

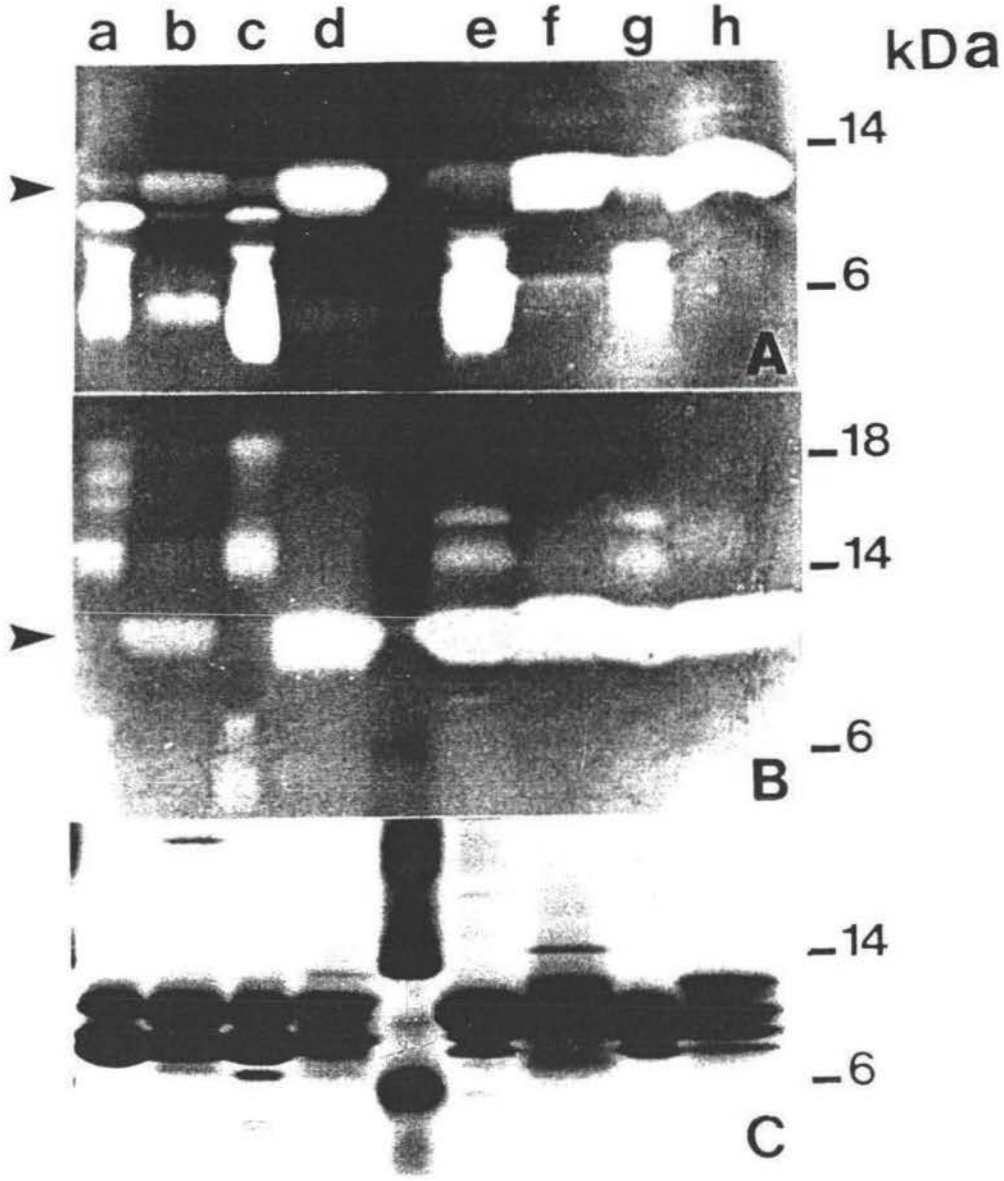


Figure 3.13 SDS-PAGE of serine proteinase inhibitors purified as peak I and II by Sephadex G-75 chromatography (see Figure 3.12A-D). Proteins were separated through a gradient (10-20%) SDS-polyacrylamide gel at 25 mA constant current for 8 h, and the separated proteins stained for inhibitory activity against chymotrypsin (**A**), trypsin (**B**), and for protein (**C**). Lane **a**, *F. arundinaceae* cv. Grasslands Garland peak I; **b**, *F. arundinaceae* cv. Grasslands Garland peak II; **c**, *F. arundinaceae* cv. Grasslands Roa peak I; **d**, *F. arundinaceae* cv. Grasslands Roa peak II; **e,** hybrid *Lolium x boucheanum* cv. Grasslands Greenstone peak I; **f**, hybrid *L. x boucheanum* cv. Grasslands Greenstone peak II; **g**, *L. perenne* cv. Grasslands Ruanui peak I; **h**, *L. perenne* cv. Grasslands Ruanui peak I. Prestained protein molecular weight standards, low range (GIBCO BRL, Life Technology, Gaithersburg, USA) were used.

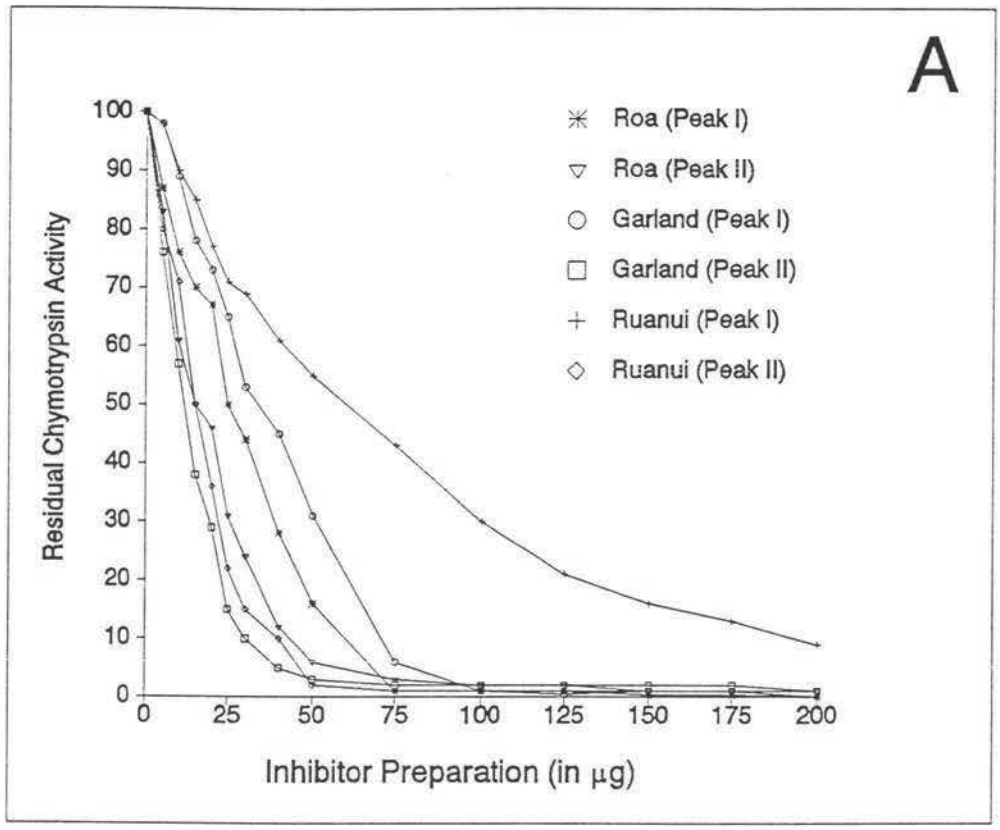
pattern was consistent across all four cultivars examined (Figure 3.13A; lanes a,c,e,g). For the two *F. arundinaceae* cultivars, a larger iso-inhibitor of *ca.* 10 kDa was also observed (Figure 3.13A; lanes a,c). Peak II from all the four cultivars demonstrated a single major iso-inhibitor of *ca.* 12 kDa (Figure 3.13A; lanes b,d,f,h; arrowed). A lower weight iso-inhibitor of about 5 kDa is present in *F. arundinaceae* cv. Grasslands Garland (Figure 3.13A; lane b), although this band did not always appear.

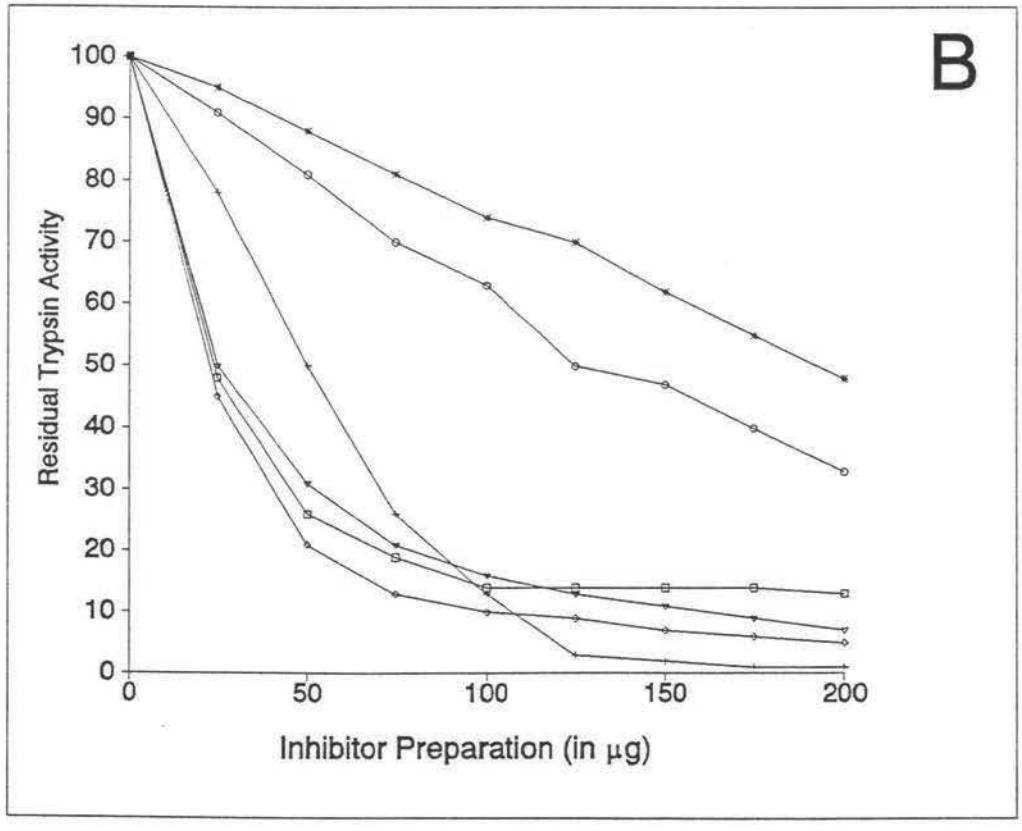
Activity staining for the presence of trypsin inhibitors once again demonstrated different banding patterns within the gel filtration fractions, although these were more genus-dependent (Figure 3.13B). A major protein band of *ca.* 12 kDa was present in both gel filtration peaks in the two *Lolium* cultivars, but only in peak II from the two *F. arundinaceae* cultivars (Figure 3.13B; lane arrowed). Peak I from the *F. arundinaceae* cultivars contained at least 6 iso-inhibitors, that had molecular weights ranging from *ca.* 3 kDa to 20 kDa (Figure 3.13B; lanes a,c). Peak I from the two *Lolium* cultivars contained two trypsin iso-inhibitors of *ca.* 15 kDa and *ca.* 16 kDa (Figure 3.13B; lanes e,g).

In comparison with the results of gel electrophoresis of the inhibitors from peak I of four cultivars from the smaller G-75 column (Figures 3.5A,B; Figures 3.8A,B), the larger column gave a better separation of proteins. In particular, peak II from all the cultivars yielded a single *ca.* 12 kDa dual trypsin/chymotrypsin inhibitor band (Figures 3.13A,B; arrowed).

To determine the potency of each inhibitor peak from three of these cultivars (excluding *Lolium* cv. Grasslands Greenstone) against trypsin and chymotrypsin, and to compare inhibitors from each genus and within a single genus, known concentrations of inhibitor preparations for each sample were titrated against a single concentration (6.25μg) of trypsin and chymotrypsin (Figures 3.14A,B). For chymotrypsin inhibition, the inhibitor preparation from peak II from all the three cultivars were similar and together were more potent than those in peak I (Figure 3.14A). Those in peak I showed differences in the level of activity, where peak 1 from cv. Grasslands Roa was the most potent, followed by cvs. Grasslands Garland and Grasslands Ruanui (Figure 3.14A).

Figure 3.14 Titration of bovine chymotrypsin (using BTpNA as substrate) (**A**), bovine trypsin (using BApNA as substrate) (**B**), with Sephadex G-75 chromatography purified inhibitor preparations (as shown in Figure 3.12A-D) from two *Festuca* cultivars and one *Lolium* cultivar. Increasing amounts of inhibitor were added to a single concentration of 0.25 mg/ml (6.25 μg per assay) chymotrypsin or trypsin and proteinase activity determined.


A
Roa (Peak I)
Roa (Peak II)
Garland (Peak I)
Garland (Peak II)
Ruanui (Peak I)
Ruanui (Peak II)
Residual Chymotrypsin Activity
Inhibitor Preparation (in µg)
0
10
20
30
40
50
60
70
80
90
100
0
25
50
75
100
125
150
175
200


B
Residual Trypsin Activity
Inhibitor Preparation (in µg)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200

For trypsin inhibition, once again, the inhibition in peak II from all the three cultivars showed similar inhibitory activity and this was also more active than that measured for peak I (Figure 3.14B). Like chymotrypsin inhibition, peak I from all the three cultivars showed different levels of activity. Among peak I, *Lolium* cv. Grasslands Ruanui contained the most active trypsin inhibitor followed by *Festuca* cvs. Grasslands Garland and Grasslands Roa (Figure 3.14B).

Comparison of this titration against chymotrypsin (Figure 3.14A) with the titration against chymotrypsin obtained previously using samples purified by the smaller G-75 column (peak I; Figure 3.7), reveals that peak I had almost similar activity for the three cultivars. However, when peak II from the larger column is compared with peak I from the smaller G-75 column, peak II from the larger column showed a two fold increase in inhibitory activity from two *Festuca* cultivars, and a 4 fold increase in inhibitory activity was observed for *Lolium* cv. Grasslands Ruanui. Peak II from these three cultivars also had good trypsin inhibitory activity. These results, together with results from gel electrophoresis (Figures 3.13A,B; arrowed) indicate that, in terms of the inhibitor, peak II contained the most pure inhibitor and probably is a dual trypsin/chymotrypsin inhibitor.

3.4.2 Anhydro-trypsin affinity chromatography of a *ca.* 12 kDa dual trypsin/chymotrypsin inhibitor from *F. arundinaceae* cultivar Grasslands Garland

It was impossible to characterise all the iso-inhibitors present in the four cultivars in the present project. In peak II of all the four cultivars one major protein of molecular weight of *ca.* 12 kDa was present that inhibited both trypsin and chymotrypsin (Figures 3.13A,B; arrowed) and so this was selected for further purification. Since it was the only iso-inhibitor present in the gel filtration peak II of all four cultivars (Figure 3.13B) to inhibit trypsin, an anhydro-trypsin affinity column was used to selectively purify the protein.

A *ca.* 12 kDa inhibitor protein was previously isolated using ion exchange chromatography from *F. arundinaceae* cv. Grasslands Garland (Sephadex G-75

gel filtered peak I) that, on the basis of 12 N-terminal amino acid sequence, found to be member of cereals α-amylase inhibitor family. Peak II after gel filtration from *F. arundinaceae* cv. Grasslands Garland was selected for affinity chromatography in order to see if (1) the inhibitor purified previously by ion exchange was identical or (2) the 12 kDa peak II inhibitor was only a minor component of the α-amylase inhibitor preparation purified by ion exchange. This purification step was also undertaken to determine if the peak II inhibitor is comprised of two separate proteins, one active against trypsin while the another one against chymotrypsin, or is a dual trypsin/chymotrypsin inhibitor. The inhibitor sample (5 mg) after Sephadex G-75 chromatography was applied to an anhydro-trypsin affinity column and bound protein eluted with low pH. Figure 3.15 shows a typical elution profile of inhibitor protein after affinity chromatography. One single protein peak was eluted that was active against both trypsin and chymotrypsin. Active fractions were pooled, equilibrated with 25 mM sodium bicarbonate and freezed dried. The inhibitor protein was separated by gel electrophoresis (17.5% SDS-PAGE) and identical gels were stained for protein, and trypsin and chymotrypsin activity. Staining of gels indicated that only a single inhibitor protein of molecular weight of *ca.* 12 kDa (Figure 3.16A) was present in the sample. Moreover, the protein inhibited both trypsin (Figure 3.16B) and chymotrypsin (Figure 3.16C) suggesting that the inhibitor had dual activity.

A series of known concentrations of the inhibitor protein were titrated against a single concentration (1 μg) of trypsin and chymotrypsin in order to check the purity of the inhibitor (Figure 3.17). While the amount of inhibitor required for 50% inhibition of chymotrypsin activity was 1.0 μg, considerably more than than that was required to inhibit trypsin by 50%. This suggests that the inhibitor protein is still not yet pure. Therefore, to further purify the inhibitor for amino acid sequencing reverse-phase column chromatography was used.

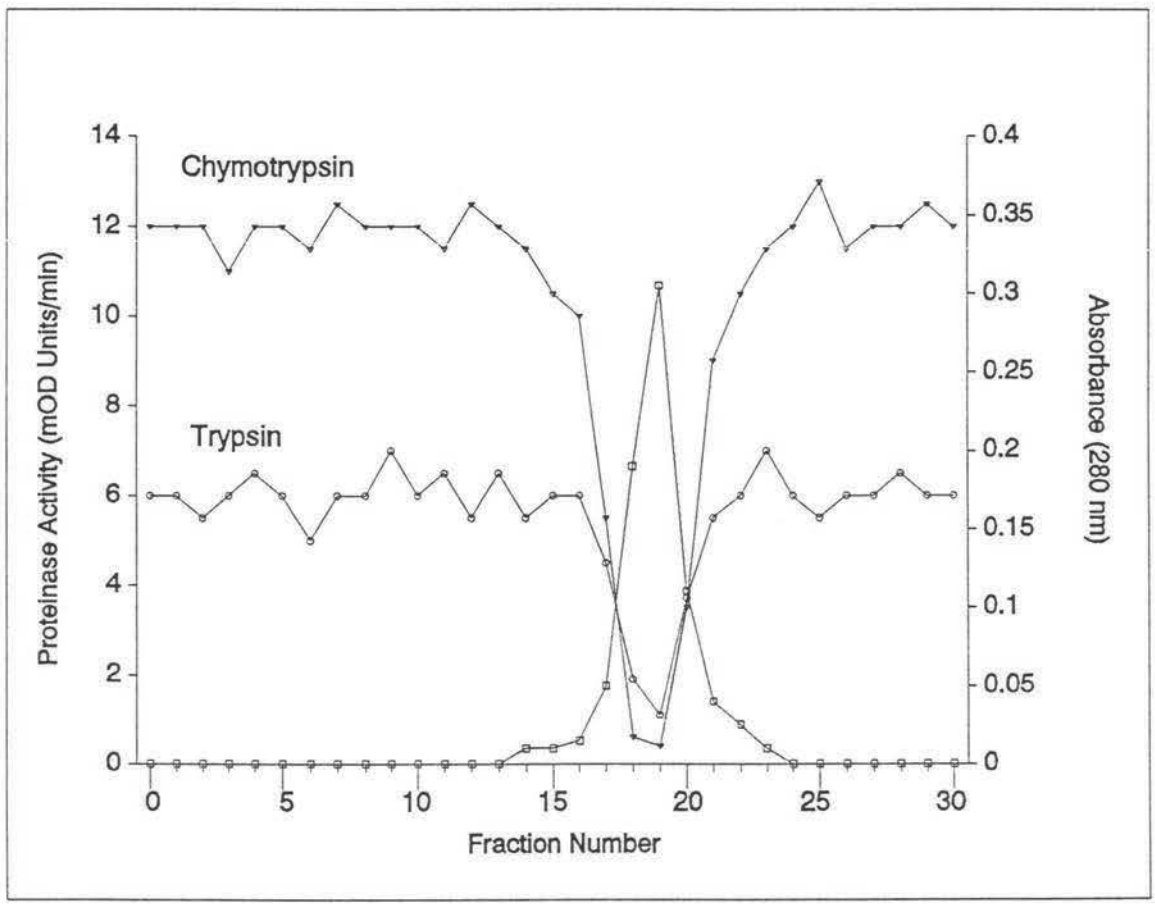
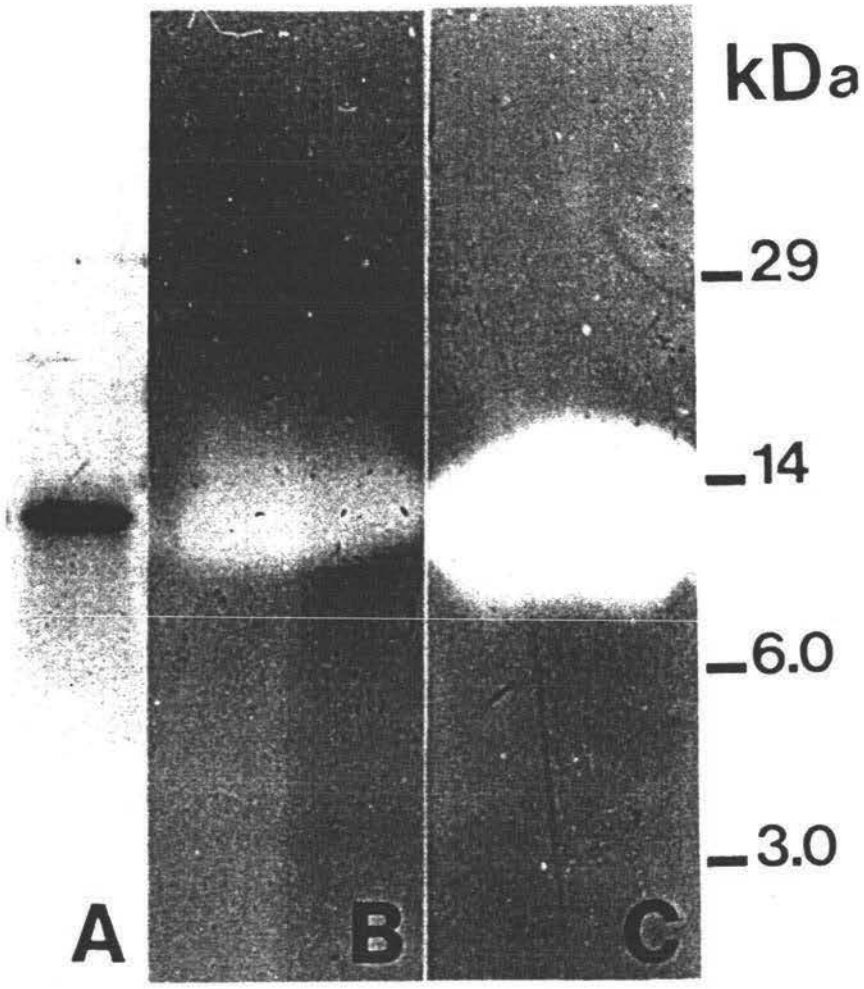
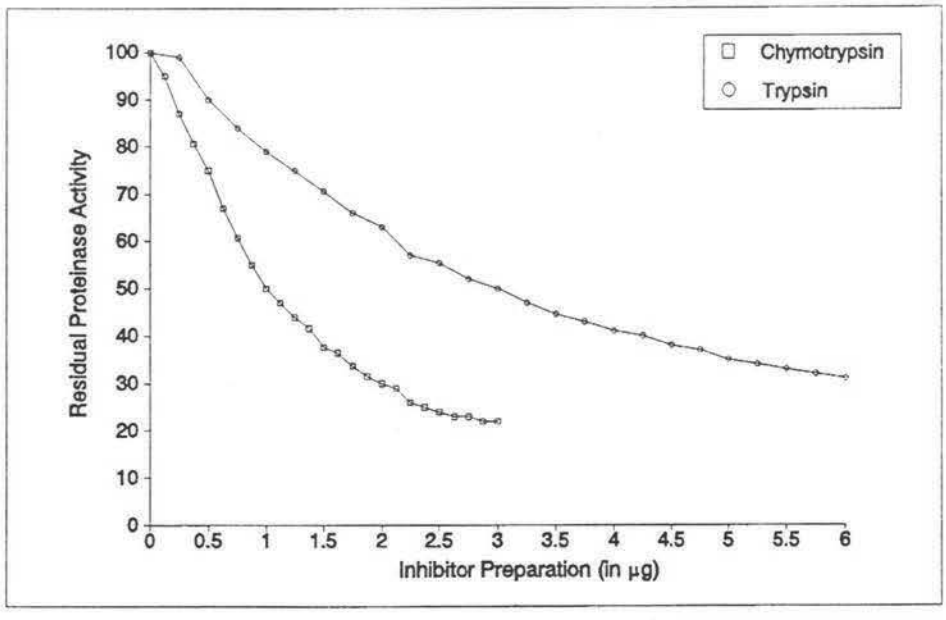


Figure 3.15 Elution of dual trypsin/chymotrypsin inhibitory activity with 50 mM HCL, 10 mM $CaCl_2$ from an anhydro-trypsin affinity column after binding gel filtration peak II from *F. arundinaceae* cv. Grasslands Garland (see Figure 3.12D) onto the column.

Figure 3.16 SDS-PAGE of a dual trypsin/chymotrypsin inhibitor from *F. arundinaceae* cv. Grasslands Garland, purified by anhydro-trypsin affinity chromatography (see section 3.4.2). Proteins were separated through a 17.5% SDS-polyacrylamide gel electrophoresis at 25 mA constant current for 6 h, and the separated proteins either stained for protein (**A**), or for inhibitory activity against trypsin (**B**) and chymotrypsin (**C**). Prestained protein molecular weight standards, low range (GIBCO BRL, Life Technology, Gaithersburg, USA) were used.

Figure 3.17 Titration of bovine chymotrypsin and bovine trypsin with the affinity-purified dual trypsin/chymotrypsin inhibitor (as shown in Figure 3.15) from *Festuca arundinaceae* cv. Grasslands Garland. Increasing amounts of inhibitor were added to a single concentration of 0.05 mg/ml (one μg per assay) chymotrypsin (□) or trypsin (○) and proteinase activity determined.


kDa
29
14
6.0
3.0
A
B
C


Chymotrypsin
Trypsin
Residual Proteinase Activity
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Inhibitor Preparation (in µg)

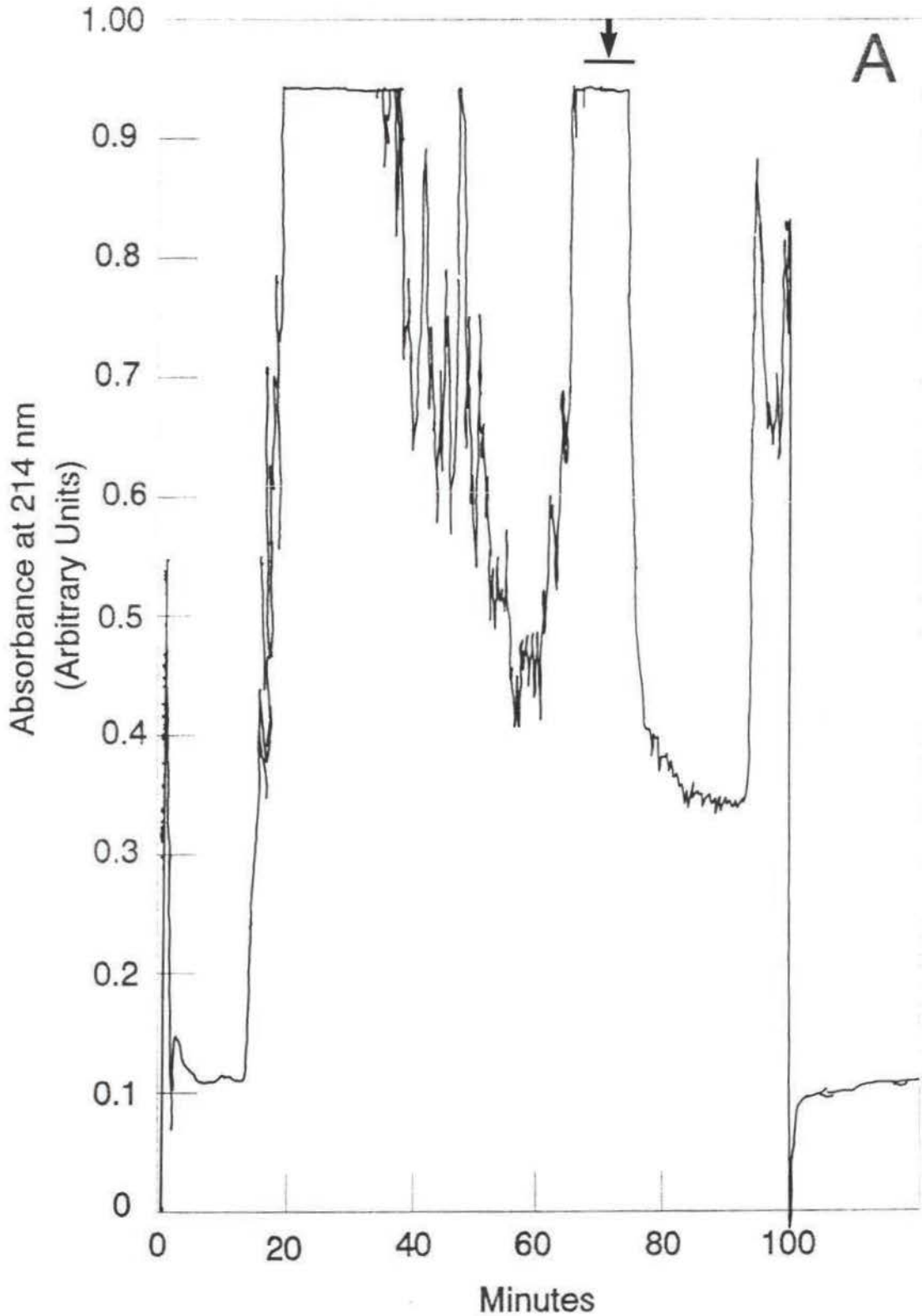
3.4.3 Reverse-phase column chromatography of the *ca.* 12 kDa dual trypsin/chymotrypsin inhibitory peptide.

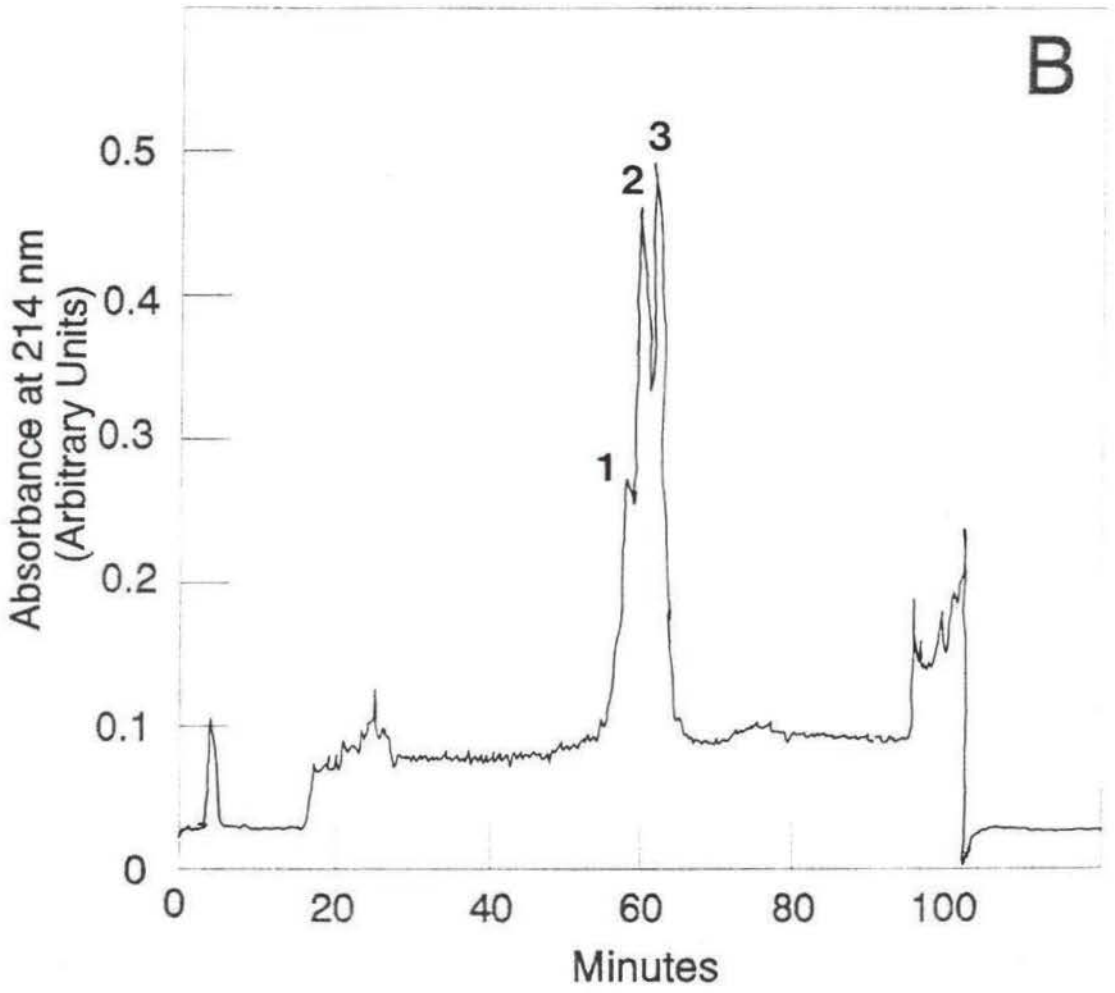
To purify the inhibitor to homogeneity, the inhibitor protein (one mg) obtained from affinity chromatography was subjected to reverse-phase HPLC. Initially, a few broad protein peaks were resolved by reverse-phase chromatography. Among these protein peaks, only a fraction of the last peak had inhibitory activity against both trypsin and chymotrypsin (Figure 3.18A; arrowed). Fractions containing proteinase inhibitory activity were pooled and re-chromatographed. For the better resolution of inhibitor proteins a different acetonitrile gradient (flattened) was used. This time three protein peaks were eluted from the column. However, only two of these (peaks 2 and 3) inhibited trypsin and chymotrypsin (Figure 3.18B; arrowed). To check the purity, the two peaks were then re-chromatographed separately (Figure 3.18CD; arrowed). Both peak 1 and 2 re-eluted as a single protein peak and contained inhibitory activity against both trypsin and chymotrypsin confirming their dual nature.

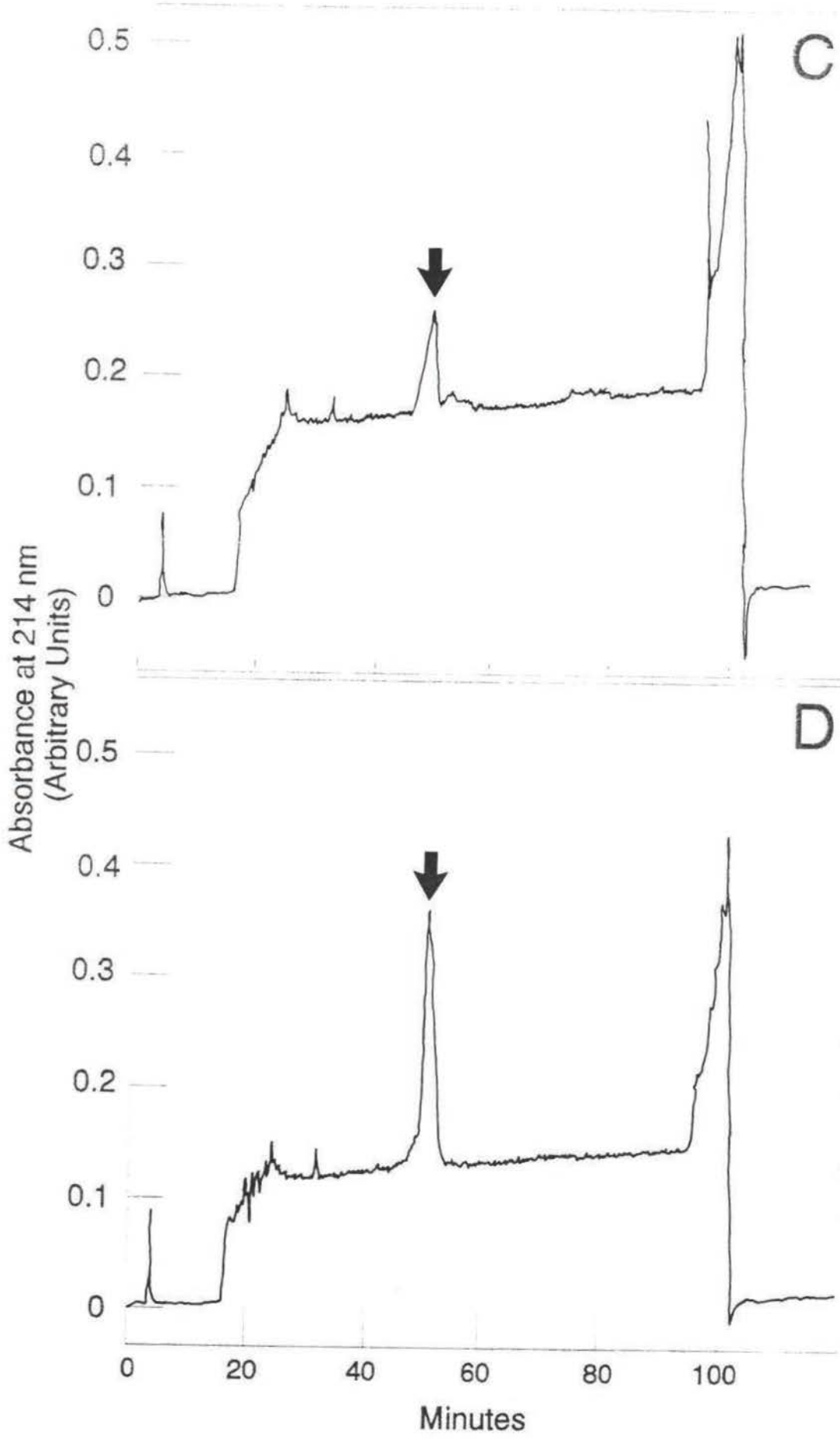
3.4.4 N-terminal amino acid sequence of reverse-phase HPLC purified inhibitor from *F. arundinaceae* cv. Grasslands Garland

The sequence of the first 20 N-terminal amino acids from both reverse-phase purified peaks was determined by Dr. C Moore, Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand. Sixteen out of the first 20 N-terminal amino acids were identified (Figure 3.19A), with both samples giving the same N-terminal amino acid sequence. The peptides were not reduced and alkylated before sequencing, and so cysteines are the most likely candidates for the missing residues. Therefore, cysteine was substituted for the missing amino acids. Two amino acid databases ["Blast-P" (Altschul *et al.*, 1990) and "Fast-A" (Pearson and Lipman, 1988)] were used to compare the alignment of the 20 amino acid sequence, including cysteine residues, with the sequence of other proteins. This search was done by Dr. N Ellison, Agresearch Grasslands, Palmerston North, New Zealand. Two proteins that had the highest consensus homology were identified and are given as

Figure 3.18 Reverse-phase column purification of affinity-purified dual trypsin/chymotrypsin inhibitor from *F. arundinaceae* cv. Grasslands Garland (as shown in Figure 3.15). **A**. Proteins were separated on a Vydac C 18 reverse-phase column with a gradient of 10% (v/v) acetonitrile, 0.1% (v/v) TFA, to 60% (v/v) acetonitrile, 0.1% (v/v) TFA. **B**. Proteinase inhibitory fractions identified in (**A**) were pooled and then subjected to reverse-phase column chromatography using the conditions as described above except that bound proteins were eluted with a different gradient, 43% (v/v) acetonitrile, 0.1% (v/v) TFA, to 60% (v/v) acetonitrile, 0.1% (v/v) TFA. Two major peaks (2 and 3) observed by Absorbance 214 nm that contained trypsin and chymotrypsin inhibitory activity. **C,D**. Peak 2 (**C**) and peak 3 (**D**) from separation (**B**) were rechromatographed separately using the conditions as described in (**A**) and (**B**) and purity of each peak was confirmed at Absorbance 214 nm and by trypsin and chymotrypsin inhibitory assay.


A
1.00
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 214 nm
(Arbitrary Units)
0
20
40
60
80
100
Minutes


B
3
2
1
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 214 nm
(Arbitrary Units)
0
20
40
60
80
100
Minutes


C
D
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 214 nm
(Arbitrary Units)
0
20
40
60
80
100
Minutes

A.

```
S V G N Q - V P G - A I P H N - L Q A -
```

B.

```
S V G N Q - V P G - A I P H N - L Q A -
• • •   •   • • •   •   • • •   • • •
S V G G Q C V P G L A M P H N P L Q A C
```

C.

```
S V G N Q - V P G - A I P H N - L Q A -
• • •         • •   • • • • •   •
S V G T S C I P G M A I P H N P L D S C
```

D.

```
S V G N Q - V P G - A I P H N - L Q A -
    •           •       •
T - G D Y F Y A G M G L P
```

Figure 3.19. A. The shared N-terminal sequence of the two dual trypsin/chymotrypsin inhibitory peptides purified from *F. arundinaceae* cv. Grasslands Garland by reverse-phase HPLC (as shown in Figures 3.16A-D). **B.** Comparison of the N-terminal sequence from the inhibitory peptides purified here with the N-terminal sequence from the trypsin inhibitor purified from rye seeds (Lyons *et al.*, 1987). **C.** Comparison with the N-terminal sequence from a dual *α*-amylase/trypsin inhibitor purified from seeds of Indian finger millet (Campos and Richardson, 1983). **D.** Comparison with chymotrypsin inhibitor purified from *F. arundinaceae* cv. Grasslands Garland by ion exchange in this study. Only one gap was extended (only in **D**) to maximise the alignment (Figure 3.11A).

Figures 3.19B,C. The first highest homology (75%) of the dual trypsin/chymotrypsin inhibitor purified in this study was with a trypsin purified from rye (*Secale cereale* L.; Lyons *et al.*, 1987; Figure 3.19B) that followed a 65% homology with a dual **α**-amylase/trypsin inhibitor purified from Indian finger millet [Ragi (*Eleusine coracana*); Campos and Richardson, 1983; Figure 3.19C]. Both inhibitors (from rye and Indian millet) belong to the barley trypsin inhibitor family (Odani *et al.*, 1983a; Garcia-Olmedo *et al.*, 1987).

Using a computer programme "Align" (Scientific and Educational Software, USA), the dual trypsin/chymotrypsin inhibitor purified here after reverse-phase chromatography (Figure 3.19A) showed only weak homology (25%; Figure 3.19D) with the chymotrypsin inhibitor purified after ion exchange (Figure 3.11A), indicating that the inhibitory peptide obtained after ion exchange was a different protein.

Chapter 4

Discussion

This project is the first attempt to investigate the spectrum of serine proteinase inhibitors that are present in dry seeds of cultivated pasture grass species. The presence of acid/heat stable chymotrypsin inhibitory activity in crude extracts of dry seeds of *Lolium perenne* (perennial ryegrass) cv. Grasslands Ruanui was previously observed by Scott and McManus (personal communication) but the inhibitor was not purified and characterized. Therefore, this project initially focused on the purification and characterization of this acid and heat stable chymotrypsin inhibitor. This inhibitor was partially purified using Sephadex G-75 gel filtration which together with SDS-PAGE suggested that the inhibitor had a native molecular weight of *ca.* 20-22 kDa (Figure 3.1). Subsequent separation using gel electrophoresis identified two inhibitory peptides of *ca.* 11 and 12 kDa (Figures 3.5A,B) which may be subunits of the mature protein. This aspect was not investigated further.

However, on the basis of these results, it is concluded that seeds of *L. perenne* cv. Grasslands Ruanui contain chymotrypsin inhibitor(s). It is most probable that similar or more potent chymotrypsin or trypsin or dual trypsin/chymotrypsin inhibitors exist in seeds of other cultivated pasture grass species. Therefore, it was decided to stop further characterisation of the chymotrypsin inhibitor from *L. perenne* cv. Grasslands Ruanui and to conduct a survey of a range of seeds from pasture grass species to see if similar or more potent chymotrypsin inhibition and/or trypsin inhibition exists in other species. If so, the intention was to compare their potency against chymotrypsin and trypsin and to purify them further.

To do this, crude extracts from seeds of 13 commercially available cultivated pasture grass cultivars were tested. Most of these were found to contain (on a comparable protein basis) chymotrypsin inhibitory activity (Table 3.1). However trypsin inhibitory activity was not detectable in these crude extracts (Table 3.1).

The object of the project was to find a potent inhibitor of trypsin and/or chymotrypsin, and so the amount of enzyme used in the survey was quite high (6.25 μg/assay). There was some trypsin inhibition observed in extracts from

all species that showed chymotrypsin inhibition, but the amount of crude protein used was comparatively high and caused high background readings. These produced a misleading estimation of trypsin inhibitory activity, and the data obtained was not consistent. Furthermore, as the criterion for finding inhibition of these proteinases was to obtain at least 50% inhibition of enzyme used, this level could not be detected for trypsin activity in extracts from any cultivar surveyed. Therefore, it was concluded that at this level of sensitivity, the seeds of all the species used in the screen do not have detectable trypsin inhibition.

Undetectable trypsin inhibitory activity could also be related to the extraction protocol as no other extraction protocols were attempted. It is possible that if a different extraction buffer and/or different extraction pH was used, trypsin inhibitory activity could have been detected. For example, trypsin inhibitor from rye was extracted using sodium acetate buffer at pH 4.9 (Boisen and Djurtoft, 1981b). A trypsin inhibitor from wheat grains has been extracted with citric buffer at pH 4.4 (Mossor and Skupin, 1990a) while another trypsin inhibitor from wheat germ was isolated with acetic acid (Odani *et al.*, 1986). Other serine proteinase inhibitors from maize, millet and rice have been extracted using NaCl (Swartz *et al.*, 1977; Shivaraj and Pattabiraman, 1980; Tashiro and Maki, 1978). A similar assumption can be made for undetectable chymotrypsin inhibitory activity in those cultivars where chymotrypsin inhibitory activity could not be detected.

Based on the results of the survey, two *Festuca arundinaceae* cultivars Grasslands Roa and Grasslands Garland that contained the most potent inhibition, and two *Lolium* cultivars, *L. perenne* cv. Grasslands Ruanui and *L. x buocheanum* cv. Greenstone that contained significant chymotrypsin inhibitory activity, were characterized further.

The presence of chymotrypsin inhibitors have been reported in seeds of members of several plant families (Richardson, 1981; Laskowski and Kato, 1980, Ryan, 1981; Garcia-Olmedo *et al.*, 1987) including members of the Graminae, particularly from seeds of almost all the cereals (Boisen, 1983). For example, barley (*H. vulgare)* seeds contain two chymotrypsin inhibitors, CI-1 (Boisen *et al.*, 1981) and CI-2 (Svendsen *et al.*, 1980). Two distinct trypsin inhibitors are also present in barley seeds, but one in endosperm (Odani *et al.*,

1983a) and the other in embryo (Boisen and Djurtoft, 1982). In pasture grass seeds used here, similar tissue specific inhibitors may be present, but only chymotrypsin inhibitory activity could be detected in the crude fractions used here.

Chymotrypsin inhibitory activity from the four cultivars selected was tested for stability at low pH (pH 3), and after heat treatment at this acidic pH. A substantial amount of inhibitory activity remained after acid and heat treatment (Table 3.5). The reasons for some loss of inhibitory activity in all the four cultivars could be (1) the presence of high phenolic (colour) compounds in the crude extract giving a false (over) estimation of chymotrypsin inhibitory activity, (2) the presence of acid/heat labile chymotrypsin inhibitors in the crude extract of seeds that were denatured during harsh treatment and resulted in less recovery of the inhibitory activity, (3) the heat treatment at low pH (pH 3) could have denatured some of the acid/heat stable inhibitor as heat treatment of proteins at very low pH (pH 3) results in the hydrolysis of aspartic acid-proline peptide bonds (M. Richardson, personal communication).

In a single tissue of a plant, there may be more than one inhibitor that is active against one or two serine proteinases. Some of these may be acid and heat stable while others may be acid/heat sensitive. For example in soybean seeds, two major serine proteinase inhibitors that belong to two distinct families of inhibitors, have been observed. The first one is the soybean (Kunitz) trypsin inhibitor (STI) that is a major trypsin inhibitor but also weakly inhibits chymotrypsin, is acid/heat sensitive and has a molecular weight of 20 kDa (Koide *et al.*, 1973). The second major inhibitor is the soybean Bowman-Birk proteinase inhibitor that inhibits both trypsin and chymotrypsin simultaneously, but is acid/heat stable and has a low molecular weight of 8 kDa (Odani and Ikenaka, 1972; Birk, 1985). In the seeds of pasture grasses used in this study, similar types of inhibitors may be present, one sensitive to acid/heat resulting in loss of half of the inhibitory activity while another is resistant to acid/heat leading to the recovery of almost all of its inhibitory activity.

To determine the reasons for the loss of inhibitory activity in pasture grass seeds, antibodies raised against purified acid/heat stable inhibitors can

be used to monitor the concentration of these inhibitors during each purification stage.

In general, though, most of the serine proteinase inhibitors purified so far from plant tissues are very stable at low pH and high temperature for several minutes (Richardson, 1977; Ryan, 1981). This property of the serine proteinase inhibitors is normally utilised as an initial step for their purification. During this step most of the storage proteins are denatured while the inhibitors still remain unaffected and active. This stability of the inhibitors involves the presence of disulphide bridges which are very prevalent in proteinase inhibitors (Ryan, 1981). For example, the stability of the soybean Bowman-Birk proteinase inhibitor has been attributed to the seven disulphide bridges (Birk, 1985). In terms of stability, the inhibitors in seeds of all the four cultivars used here are like those from foxtail millet (*S. italica*) where three types of serine proteinase inhibitors were separated after ion exchange chromatography. Of these three, two (designated FMTI-II and FMTI-III) are trypsin inhibitors and have been purified using heat treatment (80°C for 10 minutes). Like the chymotrypsin inhibitors described here, these two purified inhibitors were stable at this high temperature in acidic conditions for 10 minutes (Tashiro *et al.*, 1989; 1991).

Subjecting tissue extracts to low pH followed by heat treatment at 80°C for 10 minutes has been used as an initial purification step for the purification of inhibitor I and II from potato tubers (Melville and Ryan, 1972; Bryant *et al.*, 1976). Inhibitor I (Melville and Ryan, 1972) and inhibitor II (Bryant *et al.*, 1976) are stable when heated at high temperature (80°C) in acidic conditions (pH 3). Further a dual trypsin/chymotrypsin inhibitor from millet (*E. coracana*; ragi) seeds (Shivaraj *et al.*, 1982) and a trypsin inhibitor from wheat endosperm (Boisen and Djurtoft, 1981a) are stable at low pH (pH 2) and at high temperature (100° C for 30 minutes).

After acid/heat treatment, the inhibitors from all the four cultivars were further purified using Sephadex G-75 gel filtration chromatography. Two chymotrypsin inhibitory peaks (designated as peak I and peak II) were observed for all the four cultivars (Figures 3.6A-D) including *L. perenne* cv Grasslands Ruanui. Previously (see Figure 3.3) only one peak was eluted which corresponded to peak I from the second chromatography separation

(Figure 3.6A). Elution of only one peak in the first separation was most probably due to using less (about one third) protein in the first separation. This is confirmed in Figure 3.3 where the inhibitor peak eluted sharply at first, but then elongated indicating that peak II was probably present in the extract. However, the amount was not enough to give a sharp second peak (peak II) probably due to the less protein used.

For all the four cultivars, the inhibitory activity contained within peak I had a native molecular weight of *ca.* 20-22 kDa while that in peak II had a molecular weight of *ca.* 10-12 kDa. However, because the amount of total protein in peak II was not sufficient for further analysis, only the chymotrypsin inhibitory activity in peak I was characterised further. SDS-PAGE of peak I from the two *Lolium* cultivars revealed similar results to that obtained for the initial separation of cv. Grasslands Ruanui (Figure 3.5). The native molecular weight was *ca.* 20-22 kDa, and SDS-PAGE of this peak revealed two possible sub-units of *ca.* 11 and 12 kDa. However, SDS-PAGE of samples from two *Festuca* revealed at least six inhibitor bands that ranged in molecular weights from 3 to 12 kDa (Figur 3.8). In a titration curve, in agreement with the results obtained with crude extracts, the two *Festuca* cultivars appeared to be more potent inhibitors than those from the two *Lolium* cultivars (Figure 3.7). Therefore, to begin with, further purification of *F. arundinaceae* cv. Roa (peak I) was attempted using ion exchange chromatography. However, peak I from this cultivar did not bind to either Q-Sepharose (anionic resin exchange resin column; Tris-HCl pH 7.5) or S-Sepharose (cationic exchange resin column; sodium acetate pH 4.5), while peak I from *F. arundinaceae* cv. Grasslands Garland did bind to S-Sepharose indicating that inhibitor was positively charged at pH 4.5 (Figure 3.9).

Ion exchange chromatography has frequently been used to purify proteinase inhibitors to homogeneity and most of these inhibitors are positively charged (Melville and Ryan, 1972; Bryant *et al.*, 1976; Tashiro *et al.*, 1989; Ceciliani *et al.*, 1994; Odani *et al.*, 1983a; Warchalewski, 1987). Reasons for the failure of ion exchange chromatography of the inhibitor from cv. Grasslands Roa could firstly be an experimental error, or secondly, the inhibitor may have a different iso-electric point.

Only one inhibitor peak was eluted after ion exchange chromatography (Figure 3.9). Chymotrypsin activity staining of the SDS-PAGE indicated that the inhibitory regions on the activity gel were identical to the Sephadex G-75 purified sample, both before and after dialysis (i.e. pre-ion exchange samples; Figure 3.10). This indicates that all the inhibitors (6 inhibitors ranging in molecular weight from *ca.* 3 kDa to 12 kDa) that were present in the partially purified samples (the Sephadex G-75 purified samples) were also present in sample purified by ion exchange chromatography. However, only two major protein bands that had a molecular weight of *ca.* 11 and 12 kDa could be visualised on protein gel. This indicates that four lower molecular weight (*ca.* 3-8 kDa) inhibitory peptides were present but in very small quantity such that they could not be detected by protein staining.

The two major inhibitor protein bands were transferred to PVDF membrane and 12 N-terminal amino acid residues determined and their identity characterized (Figure 3.11A). Only the 12 kDa peptide was sufficiently pure to sequence. The highest identity (83.3%) to this protein was found with two insect **α**-amylase inhibitors, CMa from barley (*H. vulgare*) seeds (Rasmussen and Johansson, 1992; Figure 3.11B) and CM1 from wheat (*T. aestivum*) seeds (Garcia-Maroto *et al.*, 1990; Figure 3.11C). The next best identity was found with an **α**-amylase inhibitor, CM2 from wheat (*T. durum*) seeds (Gautier *et al.*, 1991; Figure 3.11D). However, all of these three are active against **α**-amylases and therefore belong to the cereal **α**-amylase inhibitor family (Garcia-Olmedo *et al.*, 1987). On the basis of very strong identity observed, the partially sequenced chymotrypsin inhibitor from *F. arundinaceae* cv. Grasslands Garland could be assigned to the cereal **α**-amylase inhibitor. However, a complete amino acid sequence is needed to confirm this alignment.

Further, in the literature, there is no single report that indicates the presence or absence of chymotrypsin inhibitory activity in the three **α**-amylase inhibitors that were identified to be homologous to the chymotrypsin inhibitor purified here. Most probably these **α**-amylase inhibitors would not be chymotrypsin inhibitors, because two of these **α**-amylase inhibitors, CM1 from wheat (Barber *et al.*, 1986a) and CMa from barley (Barber *et al.*, 1986b) have been assayed for trypsin inhibitory activity and were not found to inhibit trypsin.

Additionally, CM2 from *T. durum* has not been assayed for trypsin inhibitory activity. Because (1) the partial sequence of the inhibitor purified here had very high identity with the cereal ***α***-amylase inhibitors that do not inhibit chymotrypsin or trypsin and (2) during amino acid sequence determination, a number of other sequences were also present in the 11 kDa inhibitor protein, it was concluded that the inhibitor purified here may not be the chymotrypsin inhibitor of interest. It may be that the major protein identified by protein staining is a storage protein that either is an ***α***-amylase inhibitor or has homology with ***α***-amylase inhibitors. If so, this protein co-migrated (during gel filtration and ion exchange chromatography as well as SDS-PAGE) with the chymotrypsin inhibitory activity. Therefore, to check this, as well as to improve the separation of inhibitor proteins and to obtain a larger amount of protein for further purification, gel filtration was repeated with a larger Sephadex G-75 column.

For all the four cultivars, separation using Sephadex G-75 gel filtration (Figures 3.12A-D) confirmed previous results (Figures 3.6A-D) that indicated the two inhibitor peaks had similar molecular weights, i.e. peak I with a molecular weight of *ca.* 20-22 kDa; peak II with a molecular weight of *ca.* 10-12 kDa). Because of the better resolution of these proteins, and because some (though little) trypsin inhibitory activity was detectable in these cultivars at the crude level, trypsin inhibitory assays were also performed for each gel filtration fraction. For all four cultivars, trypsin inhibitory activity was observed and co-eluted within the same two chymotrypsin inhibitory peaks (Figures 3.12A-D). However, the trypsin inhibitory peak was smaller than the chymotrypsin inhibitory peak particularly for peak I suggesting that the chymotrypsin inhibitors were predominant.

Gel electrophoresis of peak I revealed a series of distinct chymotrypsin and trypsin inhibitors. For chymotrypsin, a range of low molecular weight (3 kDa-8 kDa) iso-inhibitors were observed (Figure 3.13A). This pattern was consistent between the two genera. For trypsin inhibitors, high molecular weight iso-inhibitors were observed (Figure 3.13B). Iso-inhibitors of molecular weight 15 kDa and 16 kDa were present in peak 1 from all the four cultivars while an additional 20 kDa inhibitor was observed for the two *Festuca* species and a 12 kDa inhibitor was observed for the two *Lolium* species (Figure 3.13B). Peak II

contained a major 12 kDa protein that was active against both trypsin and chymotrypsin and was consistent among the two genera (Figures 3.13A,B; arrowed).

The observed 12 kDa dual trypsin/chymotrypsin inhibitor (molecular weight determined using gel electrophoresis) in peak II is in good agreement with the range of molecular weights observed during gel electrophoresis for other serine proteinase inhibitors purified from members of the Graminae. These include a 12 kDa dual trypsin/chymotrypsin inhibitor from Indian finger millet (ragi; *E. coracana*; Shivaraj *et al.*, 1983) and job's tear (*Coix lacryma-jobi*; Ohtsubo *et al.*, 1989; Ary *et al.*, 1988), a 12 kDa bifunctional α-amylase/trypsin inhibitor from Indian finger millet (ragi; *E. coracana*; Manjunath *et al.*, 1983), a 12.5 kDa trypsin inhibitor from rye (*S. cereale*) endosperm (Boisen and Djurtoft, 1981b) and wheat (*T. vulgare*) endosperm (Boisen and Djurtoft, 1981a) and a 12.5 kDa trypsin inhibitor from maize (*Z. mays)* seeds (Mahoney *et al.*, 1984). However, a 14 kDa dual trypsin/chymotrypsin inhibitor from millet (*E. fruneutacea*; Udapa and Pattabiraman, 1985) and a 16 kDa trypsin inhibitor from barley (*H. distichum*; Nagasue *et al.*, 1988) seedling roots and barley embryo (*H. vulgare*; Boisen and Djurtoft, 1982) have slightly higher molecular weights and might be similar to the higher molecular weight trypsin inhibitor bands observed in peak 1 from all four cultivars.

There has also been a series of lower molecular inhibitors characterised in the Graminae. These include trypsin inhibitors from foxtail millet seeds (*S. italica*; 7.6 kDa; Tashiro *et al.*, 1990, 1991), a dual trypsin/chymotrypsin inhibitor from buck wheat (6 kDa; Ikeda and Kusano, 1983) and two chymotrypsin inhibitors (CI-1; 9 kDa and CI-2; 9.25 kDa) from barley (*H. vulgare*) seeds (Svendsen *et al.*, 1980; Boisen and Djurtoft, 1982).

These proteins may be similar to the low molecular weight chymotrypsin inhibitors observed by SDS-PAGE in peak 1 after gel filtration from all the four cultivars. Since this study used whole seeds, it is possible that they may contain solely trypsin and chymotrypsin inhibitors as well as dual trypsin/chymotrypsin inhibitors that are of different molecular weights and in differing abundance. In common with barley seeds, these may be specific to particular tissues in the seed (e.g. they occur in the endosperm or embryo).

Protein extraction from isolated endosperm and/or embryo can be used to verify which of these inhibitors is tissue specific.

The occurrence of different inhibitors with either similar or different specificities in a single tissue of plant has also been observed previously. For example, in potato tubers and potato and tomato leaves, two different inhibitors, designated inhibitor I and II belonging to two separate families have been found (Ryan, 1981). In potato tubers, on the basis of iso-electric points, at least 10 iso-inhibitors of potato inhibitor 1 have been observed (Richardson, 1977), while four iso-inhibitors of inhibitor II have been observed after ion-exchange chromatography (Bryant *et al.*, 1976). Similarly, in pasture grass seeds the iso-inhibitors observed during gel electrophoresis may be iso-inhibitors of a single inhibitor or are different inhibitors belonging to different families.

The observations that the molecular weights of the inhibitors identified by gel filtration and gel electrophoresis were conserved between the two genera (*Festuca* and *Lolium*) is supported by more recent taxonomic groupings of the Poaceae. For example, both *Festuca* and *Lolium* have been placed in the same tribe, the Poeae (Clayton and Renvoise, 1976; Watson and Dallwitz, 1992). Darbyshire and Warwick (1992) investigated the phylogeny of the genus *Festuca* from North America by using restriction endonuclease finger printing of chloroplast DNA, and found that *Festuca* and *Lolium* are very closely related genera. This close relationship between the two genera and classification of the Poaceae is supported by the results of this study that both *Festuca* and *Lolium* contained proteinase inhibitors of similar molecular weights observed by both gel filtration and SDS-PAGE.

Due to the better separation by the larger Sephadex G-75 column, a single *ca.* 12 kDa inhibitor protein was now observed predominantly in peak II, that may have previously eluted in peak I. This latter 12 kDa inhibitor was active against both trypsin and chymotrypsin, suggesting a dual inhibitor. Further, the 12 kDa inhibitor represented the major conserved protein in all four cultivars. Therefore, the 12 kDa inhibitor was the first selected for further characterisation. At this stage, it was not certain whether this 12 kDa protein is the same as the 12 kDa inhibitor purified previously by ion exchange from *F. arundinaceae* cv. Grasslands Garland and was partially sequenced.

To check whether the *ca.* 12 kDa inhibitor purified by ion exchange chromatography was the same inhibitor protein or if it was different, the protein was purified using affinity chromatography. To utilize the trypsin inhibitory activity, that was unique to this protein in peak II, an anhydro-trypsin affinity column was used for its purification.

Affinity chromatography has successfully been used for the purification of proteinase inhibitors from seeds of a number of plants (Plunkett *et al.*, 1982; Mossor and Skupin 1990a; Shivaraj *et al.*, 1982; Udapa and Pattabiraman, 1985; Odani *et al.*, 1986; Menegatti *et al.*, 1992; Broadway, 1993). Additionally, anhydro-trypsin affinity chromatography could also be used to determine if the 12 kDa inhibitor protein really is a dual inhibitor, or if the two inhibitory activities belong to two distinct proteins that co-migrate during gel filtration as well as gel electrophoresis.

Anhydro-trypsin affinity chromatography of *F. arundinaceae* cv. Grasslands Garland indicated that the inhibitor is probably a dual inhibitor with activity against trypsin as well as chymotrypsin (Figure 3.15). Gel electrophoresis gave a single band with a molecular weight of *ca.* 12 kDa, that inhibited both trypsin and chymotrypsin, suggesting that inhibitor protein may have a dual nature (Figures 3.16A,B). However, the amount of the inhibitor required for 50% inhibition of trypsin activity was more than the amount of the enzyme used in the assay (Figure 3.17). This suggests that the inhibitor protein may still not yet be pure. Titration also showed that the inhibitor had a higher affinity with chymotrypsin than trypsin. It was concluded that the inhibitor was probably a dual inhibitor because it bound to the anhydro-trypsin affinity column, but also showed strong inhibition of chymotrypsin. To confirm the dual nature of the inhibitor and to purify the inhibitor to homogeneity, reverse-phase column chromatography was used.

The affinity purified dual inhibitor from *F. arundinaceae* cv. Grasslands Garland, was purified to homogeneity using reverse phase HPLC where it was discovered that it consisted of two inhibitors (Figures 3.18A-D). Both inhibitors were dual chymotrypsin/trypsin inhibitors. Partially identity of both inhibitors was characterised using N-terminal amino acid sequence. N-terminal amino acid

sequence of 20 residues showed that both peptide proteins had an identical sequence (Figure 3.19A).

The two proteins purified here are the first serine proteinase inhibitors that have been purified and characterised from seeds of the cultivated pasture grasses. Their specificity is similar to a trypsin/chymotrypsin inhibitor that was purified from the millet, *Echinocloa fruneutacea* (Udupa and Pattabiraman, 1985). Partial N-terminal sequencing of the two proteins purified here have the highest homology (75%) with a trypsin inhibitor from rye seeds (Figure 3.19B; Lyons *et al.*, 1987). The second best homology (65%) was found with a dual **α**-amylase/trypsin inhibitor purified from Indian finger millet (Figure 3.19C; *Eleusine coracana* (Ragi); Campos and Richardson, 1983), although the protein characterised here does not appear to inhibit **α**-amylase (Richard Biggs, personal communication). The two inhibitors from rye and millet belong to the barley trypsin inhibitor family. Similarly, the two proteins purified here had good homology with other members of the barley trypsin inhibitor family such as the trypsin inhibitor from seeds of barley (*H. vulgare*; Odani *et al.*, 1983a), and a trypsin/Factor XIIA (Hageman factor) inhibitor from seeds of maize (*Zea mays*; Mahoney et al., 1984).

On the basis of partial sequence homology, two inhibitor proteins purified here can be assigned to the barley trypsin inhibitor family (Odani *et al.*, 1983a). Further, no homology was observed with members of other trypsin/chymotrypsin gene family, the Bowman-Birk inhibitors (Birk, 1985) and potato inhibitor I (Ryan, 1981). While these have been mainly characterised from the Leguminosae and Solanacae, members have also been identified in the Graminae (Odani *et al.*, 1986; Tashiro *et al.*, 1987; Svendsen *et al.*, 1980; Jonassen and Svendsen, 1982). Similarly, no homology was found with members of the white mustard/oil-rape trypsin/chymotrypsin inhibitor family (Menagatti *et al.*, 1992; Ceciliani *et al.* 1994) suggesting further that the inhibitors purified here belong to the barley trypsin inhibitors family. However, the complete amino acid sequence is needed before these two inhibitor proteins can be assigned to any family with certainty.

The partial N-terminal sequence from the protein purified by a combination of affinity and reverse-phase chromatography (Figure 3.19A) had

only 25% homology (Figure 3.19D) with that purified previously by ion exchange (Figure 3.11A). This suggests that the inhibitor purified after ion exchange was a different inhibitor from that purified by reverse-phase chromatography. The inhibitor purified by ion exchange belongs to the cereals *α*-amylase inhibitor family while the inhibitor purified after reverse-phase belongs to the barley trypsin inhibitor family. Since members of these two families have homologies with each other, some authors have suggested a new super family by combining these two together, that is designated the cereals super family. Therefore, the two inhibitor sequences obtained here are members of cereals super family (Bloch and Richardson, 1992; Garcia-Olmedo *et al.*, 1987). However, on the basis of specificity, these two can be classed as two separate families. Therefore, the ion exchange purified inhibitor could be an *α*-amylase inhibitor that has co-migrated with the chymotrypsin inhibitor during all the purification steps, but did not bind to the anhydro-trypsin affinity column. It was concluded on the basis of N-terminal amino acid sequence that the *ca.* 12 kDa chymotrypsin inhibitor purified by ion-exchange chromatography, and the *ca.* 12 kDa dual trypsin/chymotrypsin inhibitor purified by anhydro-trypsin affinity chromatography and reverse-phase chromatography are two distinct proteins. The dual trypsin/chymotrypsin inhibitor purified by affinity and reverse-phase chromatography was the serine proteinase inhibitor of interest to the current project, while the chymotrypsin inhibitor purified by ion exchange was probably not pure enough to be sequenced with certainty.

The two dual trypsin/chymotrypsin inhibitory peptides isolated in this study need further characterisation. Particularly, whether these two are different iso-inhibitors and encoded for by two different genes. The two inhibitors purified here share many properties, such as a similar molecular weight (during gel filtration, as well as gel electrophoresis of samples after gel filtration, and affinity chromatography), an identical N-terminal amino acid sequence and similar activity against trypsin and chymotrypsin (higher affinity for chymotrypsin than trypsin).

However, these two may have a slightly altered amino acid sequence, since there was a slight difference in their mobility on the reverse-phase column. A similar phenomenon has been observed for two trypsin inhibitors

purified from millet (*S. italica*). The two iso-inhibitors were isolated during ion exchange chromatography and shared many properties although they had only one amino acid difference located at their C terminal (Tashiro *et al.*, 1989; 1991). Whether a similar difference exists in the two iso-inhibitors purified here can be resolved once the complete amino acid sequence of both inhibitors has been determined.

The primary function(s) of these proteins in pasture grass seeds can be investigated. It is known for example, that seedlings of *L. perenne* establish much faster than those of *F. arundinaceae* (Langer, 1990). Similarly, there is a corresponding delay in the induction of specific enzymes (Cornford and Murray, unpublished data). Proteinase inhibitors may play a role in regulating the activity of these germination-related enzyme groups. The activity of these two inhibitors against endogenous proteinases can be studied to see if these are inhibitors of endogenous proteinases and so, involved in the control of endogenous proteinases.

Most serine proteinase inhibitors characterised from plants have been suggested to be involved in plant protection. These two inhibitors can be tested against proteinases from different insect pest of these grasses, particularly storage pests such as granary weevil (*Sitophilus granarius*), brown house moth (*Hofmannophila pseudospretella*) and white shouldered house moth (*Endrosis sarcitrella*; Somerfield, 1981) to see if their function is to protect seeds during storage against pests. As antibodies have been produced against thesetinhibitors, localisation of these inhibitors in the seed can be undertaken which may offer some clue as to their function.

Using ELISA, homology at the immunological level can be searched for with inhibitors from other cultivars of the same species, as well as other pasture grass species to see if similar inhibitors exist in seeds. Similarly, using ELISA, the dual inhibitor can be investigated in the vegetative tissues (before and after wounding) to see if the inhibitor is wound-inducible. This search can be extended to other species of *Festuca*, *Lolium* and then other genera.

References

Abe, M. and Arai, S. (1985) Purification of a cysteine proteinase inhibitor from rice, *Oryza sativa*. *Agric. Biol. Chem.* **49**: 3349-3350.

Abe, M., Arai, S., Kato, H. and Fujimaki, M. (1980) Thiol-protease inhibitor occurring in endosperm of corn. *Agric. Biol. Chem.* **44**: 685-686.

Abe, K., Emori, Y., Kondo, H., Suzuki, K. and Arai, S. (1987) Molecular cloning of a cysteine proteinase inhibitor of rice (Oryzacystatin). *J. Biol. Chem.* **262**: 16793-16797.

Adachi, T., Izumi, H., Yamada, T., Tanaka, K., Takeuchi, S., Nakamura, R. and Matsuda, T. (1993) Gene structure and expression of rice seed allergenic proteins belonging to the α-amylase/trypsin inhibitor family. *Plant Mol. Biol.* **21**: 239-248.

Altschul, S.F., Gish, W., Millar, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.

Applebaum, S.W. (1964) Physiological aspects of host specificity in the bruchidae-I. General considerations of developmental compatibility. *J. Insect Physiol.* **10**: 783-788.

Ary, M.B., Shewry, P.R. and Richardson, M. (1988) The amino acid sequence of a cereal Bowman-Birk type trypsin inhibitor from seeds of job's tears (*Coix lachryma-jobi L.*). *FEBS Letters* **229**: 111-118.

Asao, T., Imai, F., Tsuji, I., Tashiro, M., Iwami, K and Ibuki, F. (1991) Purification and characterization of a Bowman-Birk type proteinase inhibitor from faba beans (*Vicia faba* L). *Agric. Biol. Chem.* **55**: 707-713.

Atkinson, A.H., Heath,R.L., Simpson, R,J., Clarke, A.E. and Anderson, M.A. (1993) Proteinase inhibitors in *Nicotiana alata* are derived from a precursor protein which is processed into five homologous inhibitors. *The Plant Cell* **5**: 203-213

Baillargeon, M.W., Laskowski, M., Neves, D.E., Porubcan, M.A., Santini, R.E. and Markley, J.L. (1980). Soybean trypsin inhibitor (Kunitz) and its complex with trypsin. Carbon 13 nuclear magnetic resonance studies of the reactive site arginine. *Biochemistry* **19**: 5703-5710.

Balls, A.K. and Ryan, C.A. (1963) Concerning a crystalline chymotryptic inhibitor from potatoes, and its binding capacity for the enzyme. *J. Biol. Chem.* **238**: 2976-2982.

Barber, D., Sanchez-Monge, R., Garcia-Olmedo, F., Sacedo, G. and Mendez, E. (1986a) Evolution implication of sequential homologies among members of the trypsin/α-amylase inhibitor family (CM-proteins) in wheat and barley. *Biochem. Biophys. Acta* **873**: 147-151.

Barber, D., Sanchez-Monge, R., Mendez, E., Lazaro, A., Garcia-Olmedo, F. and Sacedo, G. (1986b) New α-amylase inhibitors among the CM-proteins barley (*H. vulgare*). *Biochem. Biophys. Acta* **869**: 115-118.

Barret, A.J. (1986) An introduction to the proteinases. In: *Proteinase Inhibitors*. Edited by Barret, A.J. and Salvesen, S.G. Elsevier Science, New York. Pp. 3-22.

Barret, A.J. (1987) The cystatins: a new class of peptidase inhibitors. *TIBS* **12**: 193-196.

Baumgartner, B. and Chrispeels, M.J. (1976) Partial characterisation of a protease inhibitor which inhibits the major endopeptidase present in the cotyledons of mungbeans. *Plant Physiology* **58**: 1-6.

Bidlingmeyer, U.D.V., Leary, T.R. and Laskowski, M. Jr. (1972) Identity of the tryptic and α-chymotryptic reactive sites on soybean trypsin inhibitor (Kunitz). *Biochemistry* **11**: 33033

Birk, Y. (1985) The Bowman-Birk inhibitor: trypsin and chymotrypsin-inhibitor from soybeans. *Int. J. Peptide and Protein Research.* **25**: 113-131.

Birk, Y., Gertler, A. and Khalef, S. (1963a) A pure trypsin inhibitor from soybeans. *Biochem. J.* **87**: 281-284.

Birk, Y., Gertler, A. and Khalef, S. (1963b) Separation of a *Tribolium*-protease inhibitor from soybean on a calcium phosphate column. *Biochem. Biophys. Acta* **67**: 326-328.

Bloch, C. Jr. and Richardson, M. (1992) The amino acid sequence of two 13-kDa α-amylase inhibitors from the seeds of *Sorghum bicolor* (L) Moench. *Protein Seq. Data Anal.* **5**: 27-30.

Bode, W. and Huber, R. (1992) Natural proteinase inhibitors and their interaction with proteinases. *Eur. J. Biochem.* **204**: 433-451.

Boisen, S. (1976) Characterization of the endospermal trypsin inhibitor of barley. *Phytochemistry* **15**: 641.

Boisen, S. (1983) Protease inhibitors in cereals: occurrence, properties, physiological role and nutritional influence. *Acta Agric. Scand.* **33**: 369-381.

Boisen, S., Andersen, C.Y. and Hejgaard, J. (1981) Inhibitors of chymotrypsin and microbial serine proteases in barley grains. *Physiol. Plant.* **52**: 167-176.

Boisen, S. and Djurtoft, R. (1981a) Trypsin inhibitor from wheat endosperm: purification and characterisation. *Cereal Chemistry* **52**: 460-463.

Boisen, S. and Djurtoft, R. (1981b) Trypsin inhibitor from rye endosperm: purification and properties. *Cereal Chemistry* **58**: 194-198.

Boisen, S. and Djurtoft, R. (1982) Protease inhibitor from barley embryo inhibiting trypsin and trypsin-like microbial proteases: purification and characterisation of two isoforms. *J. Sci. Food. Agric.* **33**: 431-440.

Bowman, D.E. (1946) Differentiation of soybean antitrypsin factors. *Proc. Soc. Exp. Biol. Med.* **63**: 547-550.

Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of proteins utilising the principle of protein dye binding. *Analytical Biochemistry* **72**: 248-254.

Broadway, R.M. (1993) Purification and partial characterisation of trypsin/chymotrypsin inhibitors from cabbage foliage. *Phytochemistry* **33**: 21-27.

Broadway, R.M., Duffey, S.S., Pearce, G. and Ryan, C.A. (1986) Plant proteinase inhibitors: a defense against herbivorous insects?. *Entomol. Exp. Appl.* **41**: 33-38.

Broadway, R.M., and Missurelli, D.L. (1990) Regulatory mechanisms of tryptic inhibitory activity in cabbage plants. *Phytochemistry* **29**: 3721-3725.

Brown, W.E. and Ryan, C.A. (1984) Isolation and characterisation of a wound-induced trypsin inhibitor from alfalfa leaves. *Biochemistry* **23**: 3418-3422.

Brown, W.E., Takio, K., Titani, K. and Ryan, C.A. (1985) Wound-induced trypsin inhibitor in alfalfa leaves: identity as a member of the Bowman-Birk inhibitor family. *Biochemistry* **24**: 2105-2108.

Burgess, E.P.J., Main, C.A., Stevens, P.S., Christeller, J.T., Gatehouse, A.M.R. and Laing, W.A. (1994) Effects of protease inhibitor concentration and combinations on the survival, growth and gut enzyme activities of the black field cricket, *Teleogryllus commodus*. *J. Insect Physiology* **40**: 797-802

Bryant, J., Green, T.R., Gurusaddaiah, T. and Ryan, C.A. (1976) Proteinase inhibitor II from potatoes: isolation and characterisation of its protomer components. *Biochemistry* **15**: 3418-3424.

Campos, F.A.P. and Richardson, M. (1983) The complete amino acid sequence of bifunctional α-amylase/trypsin inhibitor from seeds of ragi (Indian finger millet, *Eleusine coracana* Gaerth.) *FEBS Letters* **152**: 300-304.

Ceciliani, F., Bortolotti, F., Menegatti, E., Ronchi, S., Ascenzi, P., and Palmieri, S. (1994) Purification, inhibitory properties, amino acid sequence and identification of the reactive site of a new serine proteinase inhibitor from oil-rape (*Brassica napus*) seed. *FEBS Letters* **342**: 221-224.

Chandrasekher, G. and Pattabiraman, T. N. (1982) Natural plant enzyme inhibitors: isolation and characterization of two trypsin inhibitors from bajra (*Pennisetum typhoideum*). *Ind. J. Biochem. Biophys.* **19**: 1-7.

Clayton, W.D. and Renvoise, S.E. (1976) *Genera Graminum*. H.M. Stationery Office, London.

Cleveland, T.E., Thornburg, R.W., and Ryan, C.A. (1987) Molecular characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. *Plant Mol. Biol.* **8**: 199-207.

Cordero, M.J., Raventos, D. and SanSegundo, B. (1994) Expression of a maize proteinase inhibitor gene is induced in response to wounding and fungal infection: systemic wound-response of a monocot gene. *Plant J.* **6**: 141-150.

Cornford, C.A. and Hill, R.D. (1994) Amylase isoenzymes and BASI-like proteins in seeds of different grass species. *Seed Science Research* (in press).

Darbyshire, S.J. and Warwick, S.I. (1992) Phylogeny of North American *Festuca* (Poaceae) and related genera using chloroplast DNA restriction site variation. *Canadian J. of Botany.* **70**: 2415-2429.

Frokiaer, H., Horlyck, L. and Sorensen, S. (1994) Immunoaffinity chromatography purification and characterisation of pea trypsin inhibitors. *J. Sci. Food Agric.* **66**: 61-69.

Garcia-Maroto, F., Marana, C., Mena, M., Garcia-Olmedo, F., and Carbonero, P. (1990) Cloning of cDNA and chromosomal location of genes encoding the three types of subunits of the wheat tetrameric inhibitor of insect α-amylases. *Plant Mol. Biol.* **14**: 845-853.

Garcia-Olmedo, F., Sakedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. and Carbonero, P. (1987) Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxf. Sur. Plant Mol. and Cell Biol.* **4**: 275-334.

Gatehouse, A.M.R. and Boulter, D. (1983) Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (*Vignia unguiculata*) and other legumes on development of the bruchid beetle *Callosobruchus maculatus*. *J. Sci. Food Agri.* **34**: 345-350.

Gatehouse, A.M.R., Butler, K.J., Fenton, K.A. and Gatehouse, J.A. (1985) Presence and partial characterisation of a major proteolytic enzyme in the larval gut of *Callosobruchus maculatus*. *Entomol. Exp. Appl.* **39**: 279-286.

Gatehouse, A.M.R., Gatehouse, J.A., Dobie, P., Kilminster, A.M. and Boulter, D. (1979) Biochemical basis of insect resistance in *Vignia unguiculata*. *J. Sci. Food Agric.* **30**: 948-958.

Gautier, M.F., Alary, R., Lullien, V. and Joudrier, P. (1991) Nucleotide sequence of a cDNA clone encoding the wheat (*T. durum* Desf) CM2 protein. *Plant Mol. Biol.* **16**: 333-334.

Gennis, L.S. and Cantor, C.R. (1976) Double-headed protease inhibitors from black-eyed peas. I. Purification of two new protease inhibitors and the endogenous protease by affinity chromatography. *J. Biol. Chem.* **251**: 734-740.

Goldstein, Z., Trop, M. and Birk, Y. (1973) Multifunctional proteinase inhibitor from Bauhinia seeds. *Nature New Biol.* **246**: 29-31.

Graham, J.S., Hall, G., Pearce, G. and Ryan, C.A. (1986) Regulation of synthesis of proteinase inhibitor I and II mRNAs in leaves of wounded tomato plants. *Planta*. **169**: 399-405.

Graham, J.S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. and Ryan, C.A. (1985a) Wound-induced proteinase inhibitors from tomato leaves. I. The cDNA-deduced primary structure of preinhibitor I and its post-translational processing. *J. Biol. Chem.* **260**: 6555-6560.

Graham, J.S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. and Ryan, C.A. (1985b) Wound-induced proteinase inhibitors from tomato leaves. II. The cDNA deduced primary structure of preinhibitor II and its post-translational processing. *J. Biol. Chem.* **260**: 6561-6564.

Green, T.R. and Ryan, C.A. (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science* **175**: 776-777.

Green, T.R. and Ryan, C.A. (1973) Wound-induced proteinase inhibitor in tomato leaves: some effects of light and temperature on the wound response. *Plant Physiology* **51**: 19-21.

Gustafson, G. and Ryan, C.A. (1976) Specificity of protein turnover in tomato leaves: accumulation of proteinase inhibitors induced with the wound hormone PIIF. *J. Biol. Chem.* **25**: 7004-7010.

Hamato, N., Takano, K., Kamei-Hayashi, K. and Hara, S. (1992) Purification and characterisation of serine proteinase inhibitors from gourd (*Lagenaria leucantha* Rusby var. Gourda Makino) seeds. *Biosci. Biotech. Biochem.* **56**: 275-279.

Hara, S., Makino, J. and Ikenaka, T. (1989) Amino acid sequences and disulphide bridges of serine proteinase inhibitors from bitter gourd (*Momordica charantia* L.) seeds. *J. Biochem.* **105**: 88-92.

Harish-Kumar, P.M. Virupaksha, T.K. and Vithayathil, P.J. (1978) Sorghum proteinase inhibitors: purification and some biochemical properties. *Int. J. Peptide Protein Res.* **12**: 185-196.

Harish-Kumar, P.M. Virupaksha, T.K. and Vithayathil, P.J. (1979) Sorghum proteinase inhibitors. 2. Mode of interaction with serine proteinases. *Int. J. Peptide Protein Res.* **13**: 153-160.

Hart, P.M., Tan-Wilson, A.L. and Wilson, K.A. (1986) Proteolysis of Kunitz soybean trypsin inhibitor during germination. *Phytochemistry* **25**: 23-26.

Hass, G.M., Ager, S.P., Le Thourneau, D., Derr-Makus, J.E. and Makus, D.J. (1981) Specificity of the carboxypeptidase inhibitors from potatoes. *Plant Physiology* **67**: 754-758.

Hass, G.M., Hermodson, M.A., Ryan, C.A. and Gentry, L. (1982) Primary structures of two low molecular weight proteinase inhibitors from potatoes. *Biochemistry* **21**: 752-756.

Hass, G.M. and Ryan, C.A. (1980a) Carboxypeptidase inhibitor from ripened tomatoes: Purification and properties. *Phytochemistry* **19**: 1329-1333.

Hass, G.M. and Ryan, C.A. (1980b) Cleavage of the carboxypeptidase inhibitor from potatoes by carboxypeptidase B. *Biochem. Biophys. Res. Commun.* **97**: 1481-1486.

Hatakeyama, T, Hiraoka, M. and Funatsu, G. (1991) Amino acid sequence of the two smallest trypsin inhibitors from sponge gourd seeds. *Agric. Biol. Chem.* **55**: 2641-2642.

Hejgaard, J., Sevendsen, I.B. and Mundy, J. (1983) Barley α-amylase / subtilisin inhibitor II. N-terminal amino acid sequence and homology with inhibitors of the soybean trypsin inhibitor (Kunitz) family. *Carlsberg Res. Commun.* **48**: 91-94.

Hilder, V.A., Gatehouse A.M.R. and Boulter, D. (1990) Genetic engineering of crops for insect resistance using genes of plant origin. In: *Genetic Engineering of Crop Plants*. Edited by Lycett, G.W. and Grierson, D. Guildford, Butterworth. Pp. 51-66.

Hoff, J., Jones, C.M., Sosa, M.P. and Rodis, P. (1972) Naturally occurring crystals in the potato: isolation and identification as a protein. *Biochem. Biophys. Res. Commun.* **49**: 1525-1529.

Horiguchi, T. and Kitagishi, K. (1971) Studies on rice seed protease V. protease inhibitor in rice seed. *Plant Cell Physiology* **12**: 907-915.

Horisberger, M and Tacchini-Vonlanthen, M. (1983) Ultrastructural localization of Kunitz inhibitor on thin section of *Glycine max* (soybean) cv. maple arrow by the gold method. *Histochemistry* **77**: 37-50.

Humphries, C. (1980) Trypsin inhibitors in leaf protein concentrate. *J. Sci. Food and Agric.* **31**: 1225-1230.

Ikedo, K. and Kusano, T. (1978) Isolation and some properties of a trypsin inhibitor from buckwheat grain. *Agric. Biol. Chem.* **42**: 309-314.

Ikedo, K. and Kusano, T. (1983) Purification and properties of the trypsin inhibitors from buckwheat grain. *Agric. Biol. Chem.* **47**: 1481-1486.

Izumi, H., Adachi, T., Fujii, N., Matsuda, T., Nakamura, R., Tanaka, K., Urisu, A. and Kurosawa, Y. (1992) Nucleotide sequence of a cDNA clone encoding a major allergenic protein in rice seeds. *FEBS Letters* **302**: 213-216.

Jiao, J., Yee, C.B., Kobrehel, K. and Buchanan, B.B. (1992) Effect of thioredoxin-linked reduction on the activity and stability of the Kunitz and Bowman-Birk soybean trypsin inhibitor proteins. *J. Agric. Food. Chem.* **40**: 2333-2336.

Johns, D.C. (1986) Evaluation of the nutritional value of ryegrass-white clover leaf protein concentrate. *NZ J. Agric. Res.* **29**: 249-256.

Johnson, R., Narvaez, J. An, G. and Ryan, C.A. (1989) Expression of proteinase inhibitors I and II in transgenic tobacco plants: effect on natural defense against *Munduca sexta* larvae. *Proc. Natl. Acad. Sci. USA*. **86**: 9871-9875.

Jonassen, I.B., Ingversen, J. and Brandt, A. (1981) Synthesis of SP II albumin, β-amylase and chymotrypsin inhibitor CI-1 on polysomes from the endoplasmic reticulum of barley endosperm. *Carlsberg Res. Commun.* **46**: 175-181.

Jonassen, I.B. and Svendsen, I.B. (1982) Identification of the reactive sites in two homologous serine proteinase inhibitors isolated from barley. *Carlsberg. Res. Commun.* **47**: 199-203.

Joubert, F.J. (1984) Trypsin inhibitor from *Momordica repens* seeds. *Phytochemistry* **23**: 1401-1406.

Kashlan and Richardson (1981) The complete amino acid sequence of a major wheat protein inhibitor of α-amylase. *Phytochemistry* **20**: 1781-1784.

Keil, M., Sanchez-Serrano, J., Schell, J. and Willmitzer, L. (1986) Primary structure of a proteinase inhibitor II gene from potato (*Solanum tuberosum*). *Nuc. Acid Res.* **14**: 5641-5650.

Keilova, H. and Tomasek, V. (1976a) Isolation and some properties of cathepsin D inhibitor from potatoes. *Collection Czechoslov. Chem. Commun.* **41**: 489-497.

Keilova, H. and Tomasek, V. (1976b) Further characterisation of cathepsin D inhibitor from potatoes. *Collection Czechoslov. Chem. Commun.* **41**: 2440-2447.

Kirsi, M. and Mikola, J. (1977) Occurrence and heterogeneity of chymotrypsin inhibitors in vegetative tissues of barley. *Physiol. Plant.* **39**: 110-114.

Kirsi, M. and Mikola, J. (1971) Occurrence of proteolytic inhibitors in various tissues of barley. *Planta* **96**: 281-291.

Kiyohara, T. and Iwasaki, T. (1985a) Chemical and physicochemical characterization of the permanent and temporary trypsin inhibitors from buckwheat. *Agric. Biol. Chem.* **49**: 589-594.

Kiyohara, T. and Iwasaki, T. (1985b) Purification and properties of trypsin inhibitors from buckwheat seeds. *Agric. Biol. Chem.* **49**: 581-588.

Koide, T. and Ikeneka, T. (1973a) Studies on soybean trypsin inhibitors. 1. Fragmentation of soybean trypsin inhibitor (Kunitz) by limited proteolysis and by chemical cleavage. *Eur. J. Biochem.* **32**: 401-407.

Koide, T. and Ikeneka, T. (1973b) Studies on soybean trypsin inhibitors. 3. Amino acid sequence of the carboxyl terminal region and the complete amino acid sequence of soybean trypsin inhibitor (Kunitz). *Eur. J. Biochem.* **32**: 417-431.

Koide, T., Tsunazava, S. and Ikenaka, T. (1973) Studies on soybean trypsin inhibitors. 2. Amino acid sequence around the reactive site of soybean trypsin inhibitor. *Eur. J. Biochem.* **32**: 551-557.

Kollipara, K.P. and Hymowitz, T. (1992) Characterization of trypsin and chymotrypsin inhibitors in the wild perennial *Glycine* species. *J. Agric. Food Chem.* **40**: 2356-2363.

Kortt, A.A. (1979) Isolation and characterisation of the trypsin inhibitors from winged bean seed (*Psophocarpus tetragonolobus* (L) Dc). *Biochim. Biophys. Acta* **577**: 371-382.

Kowalski, D. and Laskowski, M. Jr. (1972) Inactivation of enzymatically modified trypsin inhibitors upon chemical modification of the α-amino group in the reactive site. *Biochemistry* **11**: 3451-3459

Kowalski, D. and Laskowski, M. (1976a) Chemical-enzymatic replacement of Ile-64 in the reactive site of soybean trypsin inhibitor (Kunitz). *Biochemistry* **15**: 1300-1309.

Kowalski, D. and Laskowski, M. (1976b) Chemical-enzymatic insertion of an amino acid residue in the reactive site of soybean trypsin inhibitor (Kunitz). *Biochemistry* **15**: 1309-1315.

Kuo, T., Pearce, G. and Ryan, C.A. (1984) Isolation and characterization of proteinase inhibitor I from etiolated tobacco leaves. *Arch. Biochem. Biophys.* **230**: 504-510.

Kunitz, M. (1945) Crystallisation of a trypsin inhibitor from soybean. *Science* **101**: 668-669.

Kunitz, M. (1946) Crystalline soybean trypsin inhibitor. *J. Gen. Physiol.* **29**: 149-154.

Kunitz, M. (1947a) Crystalline soybean trypsin inhibitor. 2. General properties. *J. Gen. Physiol.* **30**: 291-307.

Kunitz, M. (1947b) Isolation of a crystalline protein compound of trypsin and trypsin inhibitor. *J. Gen. Physiol.* **30**: 311-320.

Kunitz, M. (1949) The kinetics and thermodynamics of reversible denaturation of crystalline trypsin inhibitor. *J. Gen. Physiol.* **32**: 241-263.

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Langer, R.H.M. (1990) *Pastures: their ecology and management.* Oxford University Press, Oxford.

Laskowski, M., Jr. (1986) Protein inhibitors of serine proteinase-mechanism and classification. In: *Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods.* Edited by Friedman, M. Plenum Publishing, New York. Pp. 1-17.

Laskowski, M. Jr. and Kato, I. (1980) Protein inhibitors of proteinases. *Ann. Rev. Biochem.* **49**: 593-626.

Lee, J.S., Brown, W.E., Graham, J.S., Pearce, G., Fox, E.A., Dreher, T.W., Ahern, K.G., Pearson, G.D. and Ryan, C.A. (1986) Molecular characterisation and phylogenetic studies of a wound-inducible proteinase inhibitor I gene in *Lycopersicon* species. *Proc. Natl. Acad. Sci. USA.* **83**: 7277-7281.

Liener, I.E. and Kakade, M.L. (1980) Protease inhibitors. In: *Toxic Constituents of Plant Foodstuffs.* 2nd ed. Edited by Liener, I.E. Academic Press, New York. Pp. 7-71.

Lipke, H., Frankel, G.S. and Liener, I.E. (1954) Growth inhibitors: effect of soybean inhibitors on growth of *Tribolium confusum. J. Agric. Food Chem.* **2**: 410-414.

Lyons, A., Richardson, M., Titham, A.S. and Shewry, P.R. (1987) Characterization of homologous inhibitors of trypsin and α-amylase from seeds of rye (*Secale cereale* L.). *Biochim. Biophys. Acta* **915**: 305-313.

Madl, R.L. and Tsen, C.C. (1974) Trypsin and chymotrypsin inhibitors of triticale. In: *Triticale; first man-made cereal.* Edited by Tsen, C.C. American Association of Cereal Chemists, Minnesota. Pp. 168-182.

Mahoney, W.C., Hermodson, M.A., Jones, B., Powers, D.D., Corfman, R.S. and Reeck, G.R. (1984) Amino acid sequence and secondary structural analysis of the corn inhibitor of trypsin and activated Hageman factor. *J. Biol. Chem.* **259**: 8412-8416.

Maki, Z., Tashiro, M., Sugihara, N. and Kanamori, M. (1980) Double headed nature of a trypsin inhibitor from rice bran. *Agric. Biol. Chem.* **44**: 953-955.

Manen, J.F., Simon, P., Slooten, J.C.V., Osteras, M., Frutiger, S. and Hughes, G.J. (1991) A nodulin specifically expressed in senescent nodules of winged bean is a protease inhibitor. *The Plant Cell* **3**: 259-270.

Manjunath, N. H., Veerabhadrappa, P. and Virupaksha, T. K. (1983) Isolation and characterization of a trypsin inhibitor from finger millet. *Phytochemistry* **22**: 2349-2357.

Matsuo, M., Hamato, N., Takano, R., Kamei-Hayashi, K., Yasuda-Kamatani, K., Nomoto,K., and Hara, S. (1992) Trypsin inhibitors from bottle gourd (*Lagenaria leucantha* Rusby var. depressa makino) seeds. Purification and amino acid sequences. *Biochim. Biophys. Acta* **1120**: 187-192.

Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**: 10035-10038.

McGuri, B., Pearce, G., Orozco-Cardenas, M. and Ryan, C.A. (1992) Structure, expression, and antisense inhibition of the systemin precursor gene. *Science* **255**: 1570-1573.

McManus, M.T., Laing, W.A. and Christeller, J.T. (1994a) Wounding induces a series of closely-related trypsin/chymotrypsin inhibitory peptides in leaves of tobacco. *Phytochemistry* (In press).

McManus, M.T., Laing, W.A. Christeller, J.T. and White, D.W.R. (1994b) Post-translational modification of an iso-inhibitor from the potato inhibitor II gene family in transgenic tobacco yields a peptide with homology to chymotrypsin inhibitor I (PCI-I). *Plant Physiology* (In press).

McManus, M.T., White, D.W.R. and McGregor, P.G. (1994c) Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect growth of insect pests. *Transgenic Research* **3**: 50-58.

Melville, J. and Ryan, C.A. (1972) Chymotrypsin inhibitor I from potatoes: large scale preparation and characterisation of its subunit components. *J. Biol. Chem.* **247**: 3445-3453.

Menegatti, E., Tedechi, G., Ronchi, S., Bortolotti, F., Ascenzi, P., Thomas, R.M., Bolognesi, M. and Palmieri, S. (1992) Purification, inhibitory properties and amino acid sequence of a new serine proteinase inhibitor from white mustard (*Sinapis alba* L.) seed. *FEBS Letters* **301**: 10-14.

Mikel, C.E. and Standish, J. (1947) Susceptibility of processed soy flour and soy grits in storage to attack by *Tribolium castaneum* (Herbst). *Univ. Minn. Agricult. Exp. Stat. Tech. Bull.* **178**: 1-20.

Mikola, J. and Kirsi, M. (1972) Differences between endospermal and embryonal trypsin inhibitors in barley, wheat and rye. *Acta Chem. Scand.* **26**: 787-795.

Mikola, J. and Suolinna, E.M. (1971) Purification and properties of an inhibitor of microbial alkaline proteinases from barley. *Arch. Biochem. Biophys.* **144**: 566-575.

Mikola, J. and Suolinna, E.M. (1969) Purification and properties of a trypsin inhibitor from barley. *Eur. J. Biocem.* **9**: 555-560

Moralejo, M.A., Garcia-Casado, G., Sanchez-Monge, R., Lopez-Otin, C., Romagosa, I., Molina-Cano, J.L. and Salcedo, G. (1993) Genetic variants of the trypsin inhibitor from barley endosperm show different inhibitory activities. *Plant Science* **89**: 23-29.

Mossor, G. and Skupin, J. (1990a) Isolation and identification of trypsin inhibitor from wheat grain, Beta variety. *Die Nahrang* **34**: 151-123.

Mossor, G. and Skupin, J. (1990b) Biochemical characteristics of trypsin inhibitor from wheat grain, Beta variety. *Die Nahrang* **34**: 431-438.

Nagasue, A., Fukamachi, H., Ikenaga, H. and Funatsu, G. (1988) The amino acid sequence of barley roolet trypsin inhibitor. *Agric. Biol. Chem.* **52**: 1505-1514.

Nelson, C.E. and Ryan, C.A. (1980) *In vitro* synthesis of pre-proteins of vacuolar compartmented proteinase inhibitors that accumulate in leaves of wounded tomato plants. *Proc. Natl. Acad. Sci. USA.* **77**: 1975-1979.

Nishino, J., Takano, R., Kamei-Hayashi, K., Minakata, H., Nomoto, K. and Hara, S. (1992) Amino acid sequence of trypsin inhibitors from oriental pickling melon (*Cucumis mel* L. var. Conomon Makino) seeds. *Biosci. Biotech. Biochem.* **56**: 1241-1246.

Obidairo, T. K. and Obasuyi, H. N. (1991) Characterization of trypsin inhibitor from millet (*Pennisetum typhoideum*). *Pak. J. Sci. Ind. Res.* **34**: 127-130.

Odani, S. and Ikeneka, T. (1972) Studies on soybean trypsin inhibitor IV. Complete amino acid sequence and the antiproteinase sites of Bowman-Birk soybean proteinase inhibitor. *J. Biochem.* **71**: 839-848.

Odani, S. and Ikeneka, T. (1973a) Studies on soybean trypsin inhibitor VIII. Disulphide bridges in soybean Bowman-Birk inhibitor. *J. Biochem.* **74**: 697-715.

Odani, S. and Ikeneka, T. (1973b) Scission of soybean Bowman-Birk proteinase inhibitor into two small fragments having either trypsin or chymotrypsin inhibitory activity. *J. Biochem.* **74**: 857-860.

Odani, S., Koide, T. and Ono, T. (1983a) The complete amino acid sequence of barley trypsin inhibitor. *J. Biol. Chem.* **258**: 7998-8003.

Odani, S., Koide, T. and Ono, T. (1986) Wheat germ trypsin inhibitors. Isolation and structural characterization of single-headed and double-headed inhibitors of the Bowman-Birk type. *J. Biochem.* **100**: 975-983.

Odani, S., Koide, T., Ono, T. and Ohnishi, K. (1983b) Structural relationship between barley (*Hordeum vulgare*) trypsin inhibitor and castor-bean (*Ricinus communis*) storage protein. *Biochem. J.* **213**: 543-545.

Ohtsubo, K. (1985) Properties of a trypsin inhibitor from job's-tears (*Coix lacryma-jobi L.* var. Ma-yuen Stapf). *Agric. Biol. Chem.* **49**: 1985-1991.

Ohtsubo, K. (1989) Effects of salts, protease digestions, chemical modifications, and heat treatment on the trypsin inhibitory activity of the protease inhibitor from job's-tears (*Coix lacryma-jobi L.* var. Ma-yuen Stapf). *Agric. Biol. Chem.* **53**: 333-339.

Ohtsubo, K., Harada, K. and Kawasaki, S. (1989) Changes in levels of proteases and their inhibitors during development of the seeds of job's tears (*Coix lacryma-jobi L.* var. Ma-yuen Stapf) and their localization and interactions. *Plant Cell Physiology* **30**: 699-705.

Ozawa, K. and Laskowski, M. Jr. (1966) The reactive site of trypsin inhibitors. *J. Biol. Chem.* **241**: 3955-3961.

Papastoitsis, G. and Wilson, K.A. (1991) Initiation of the degradation of the soybean Kunitz and Bowman-Birk trypsin inhibitors by a cysteine protease. *Plant Physiology* **96**: 1086-1092.

Pearce, G., Johnson, S. and Ryan, C.A. (1993) Purification and characterization from tobacco (*Nicotiana tobacum*) leaves of six small, wound-inducible proteinase iso-inhibitors of the potato inhibitor II family. *Plant Physiology* **102**: 634-644.

Pearce, G., Ryan, C.A. and Liljegren, D. (1988) Proteinase inhibitors I and II in fruit of wild tomato species: transient components of a mechanism for defense and seed dispersal. *Planta* **175**: 527-531.

Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* **253**: 895-897.

Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA.* **85**: 2444-2448.

Peatot, V., Holzer, F.M. and Walling, L.L. (1991) Differential expression of tomato proteinase inhibitor I and II genes during bacterial pathogen invasion and wounding. *Molecular Plant-Microbe Interactions* **4**: 284-292.

Plunkett, G., Senear, D.F., Zuroske, G. and Ryan, C.A. (1982) Proteinase inhibitors I and II from leaves of wounded tomato plants purification and properties. *Arch. Biochem. Biophys.* **213**: 463-472.

Poerio, E., Carrano, L., Garzillo, A.M. and Bounocore, V. (1989) A trypsin inhibitor from the water-soluble protein fraction of wheat kernel. *Phytochemistry* **28**: 1307-1311.

Rackis, J.J. and Anderson, R.L. (1964) Isolation of four soybean trypsin inhibitors by DEAE-cellulose chromatography. *Biochem. Biophys. Res. Commun.* **15**: 230-235.

Rancour, J.M. and Ryan, C.A. (1968) Isolation of carboxypeptidase B inhibitor from potatoes. *Arch. Biochem. Biophys.* **125**: 380-382.

Rasmussen, S.K. and Johansson, A. (1992) Nucleotide sequence of a cDNA coding for the barley seed protein CMa: an inhibitor of insect α-amylase. *Plant Mol. Biol.* **18**: 423-427.

Rasmussen, U., Munck, L. and Ullrich. S.E. (1990) Immunogold localization of chymotrypsin inhibitor-2, a lysine-rich protein, in developing barley endosperm. *Planta* **180**: 272-277.

Reddy, M.N., Keim, P.S., Heinrikson, R.L. and Kizdy, F.J. (1975) Primary structural analysis of sulfhydryl protease inhibitors from pineapple stem. *J. Biol. Chem.* **250**: 1741-1750.

Richardson, M. (1977) The proteinase inhibitors of plants and micro-organisms. *Phytochemistry* **16**: 159-169.

Richardson, M. (1979) The complete amino acid sequence and the trypsin reactive (inhibitory) site of the major proteinase inhibitor from the fruit of the aubergine (*Solanum melongena* L.). *FEBS Letters* **104**: 322-326.

Richardson, M. (1981) Protein inhibitors of enzymes. *Food Chemistry* **6**: 235-253.

Richardson, M., Valdes-Rodriguez, S. and Blanco-Labra, A. (1987) A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. *Nature* **327**: 432-434.

Rodis, P. and Hoff, J.E. (1984) Naturally occurring protein crystals in the potato, inhibitor of papain, chymopapain and ficin. *Plant Physiology* **74**: 907-911.

Rodriguez-Palenzuela, P., Royo, J., Gomez, L., Sanchez-Monge, R., Salcedo, G., Molina-Cano, J.L., Garcia-Olmado, F. and Carbonero, P. (1989) The gene for trypsin inhibitor CMe is regulated trans by the lys 3a locus in the endosperm of barley (*Hordeum vulgares* L.). *Mol. Gen. Genet.* **219**: 474-479.

Ross, C.W. and Detling, J.K. (1983) Investigations of trypsin inhibitors in leaves of four North American prairie grasses. *J. Chem. Ecol.* **9**: 247-257.

Royer, A., Miege, M.M., Grange, A., Meige, J. and Mascherpa, J.M. (1974) Inhibitors antitrypsine et activités protéolytiques des albumines de grain de *Vigna unguiculata*. *Planta* **119**: 1-16.

Ryan, C.A. (1968) Synthesis of chymotrypsin inhibitor I protein in potato leaflets induced by detachment. *Plant Physiology* 43:1859-1865.

Ryan, C.A. (1973) Proteolytic enzymes and their inhibitors in plants. *Ann. Rev. Plant Physol.* **24**: 174-196.

Ryan, C.A. (1974) Assay and biochemical properties of the proteinase inhibitor-inducing factor, a wounding hormone. *Plant Physiol.* **54**: 328-332.

Ryan, C.A. (1978) Proteinase inhibitors in plants leaves: a biochemical method for pest-induced natural plant protection. *TIBS* **5**: 148-150.

Ryan, C.A. (1981) Proteinase inhibitors. In: *The Biochemistry of Plants.* Vol. **6**. Academic, New York. Pp. 351-370.

Ryan, C.A. (1984) Defense responses of plants. In: *Plant Gene Research: Genes Involved in Microbe Plant Interactions.* Edited by Verma, D.P.S. and Hohn, T.H. Springer Verlag, New York. Pp. 375-386.

Ryan, C.A. (1989) Proteinase inhibitor gene families: strategies for transformation to improve plant defense against herbivores. *BioEssays* **10**: 20-24.

Ryan, C.A. (1990) Protease inhibitors in plant: genes for improving defenses against insect and pathogens. *Ann. Rev. Phytopathology* **28**: 425-449.

Ryan, C.A., Hass, G.M. and Kuhn, R.W. (1974) Purification and properties of a carboxypeptidase inhibitor from potatoes. *J. Biol. Chem.* **249**: 5495-5499.

Ryan, C.A., Huisman, O.C. and Van-Denburgh, R.W. (1968) Transitory aspects of a single protein in tissues of *Solanum tuberosum* and its coincidence with the establishment of new growth. *Plant Physiol.* **43**: 589-596.

Ryan, C.A., Kuo, T., Pearce, G. and Kunkel, R. (1976) Variability in the concentration of three heat stable proteinase inhibitor proteins in potato tubers. *Am. Potato J.* **53**: 443-455.

Sanchez-Serrano, J., Schmidt, R., Schell, J. and Willmitzer, L. (1986) Nucleotide sequence of proteinase inhibitor II gene encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. *Mol. Gen. Genet.* **203**: 15-20.

Sealock, R.W. and Laskowski, M. (1969) Enzymatic replacement of arginyl by a lysyl residue in the reactive site of soybean trypsin inhibitor. *Biochemistry* **8**: 3703-3710.

Seemuller, V., Eulitz, M., Fritz, H. and Strobl, A. (1980) Structure of the elastase-cathepsin G inhibitor of the Leech *Hirudo medicinalis*. *Hoppe-Seyler's Z. Physiol. Chem.* **361**: 1841-1846.

Shain, Y. and Mayer, A.M. (1965) Proteolytic enzymes and endogenous trypsin inhibitor in germinating lettuce seeds. *Physiol. Plant.* **18**: 853-859.

Shain, Y. and Mayer, A.M. (1968) Activation of enzymes during germination: trypsin-like enzyme in lettuce. *Phytochemistry* **7**: 1491-1498.

Shivaraj, B. and Pattabiraman, T. N. (1980) Natural plant enzyme inhibitors: part VIII- purification and properties of two α-amylase inhibitors from ragi (*Eleusine coracana*) grains. *Ind. J. Biochem. Biophys.* **17**: 181-185

Shivaraj, B. and Pattabiraman, T. N. (1981) Natural plant enzyme inhibitors: characterisation of an unusual α-amylase / trypsin inhibitor from ragi (*Eleusine coracana* Geartn.). *Biochem. J.* **193**: 29-36.

Shivaraj, B., Roa, N. and Pattabiraman, T. N. (1982) Natural plant enzyme inhibitors: isolation of a trypsin / α-amylase inhibitor and a chymotrypsin / trypsin inhibitor from ragi (*Eleusine coracana*) grains by affinity chromatography and study of their properties. *J. Sci. Food Agric.* **33**: 1080-1091.

Shumway, L.K., Rancour, J.M. and Ryan, C.A. (1970) Vacuolar protein bodies in tomato leaf cells and their relationship to storage of chymotrypsin inhibitor I protein. *Planta*. **93**: 1-14.

Shumway, L.K., Yang, V.V. and Ryan, C.A. (1976) Evidence for the presence of proteinase inhibitor in vacuolar protein bodies of plant cells. *Planta* **129**: 161-165.

Somerfield, K.G. (1981) Insects and mites: in commercial grain and seed stores: identification. *Farm Production and Practice*. FPP **369**: 26-30

Sosulski, F.W., Minja, L.A. and Christensen, D.A. (1988) Trypsin inhibitors and nutritive value in cereals. *Plant Foods for Human Nutrition* **38**: 23-34.

Steffens, R., Fox, F.R. and Kassel, B. (1978) Effect of trypsin inhibitors on growth and metamorphosis of corn borer larvae *Ostrina nubilalis*. *J. Agric. Food Chem.* **26**: 170-175.

Svendsen, I.B., Boisen, S. and Hejgaard, J. (1982) Amino acid sequence of serine proteinase inhibitor CI-1 from barley: homology with barley inhibitor CI-2, potato inhibitor I and leech eglin. *Carlsberg Res. Commun.* **47**: 45-53.

Svendsen, I.B., Hejgaard, J. and Chavan, J.K. (1984) Subtilisin inhibitor from seeds of broad bean (*Vicia faba*): purification, amino acid sequence and specificity of inhibition. *Carlsberg Res. Commun.* **49**: 493-502.

Svendsen, I.B., Hejgaard, J. and Mundy, J. (1986) Complete amino acid sequence of the α-amylase / subtilisin inhibitor from barley. *Carlsberg Res. Commun.* **51**: 43-50.

Svendsen, I.B., Jonassen, I.B., Hejgaard, J. and Boisen, S. (1980) Amino acid sequence homology between a serine protease inhibitor from barley and potato inhibitor I. *Carlsberg Res. Commun.* **45**: 389-395.

Swartz, M.J. Mitchell, H.L., Cox, D.J. and Reeck, G.R. (1977) Isolation and characterization of trypsin inhibitor from opaque-2 corn seeds. *J. Biol. Chem.* **252**: 8105-8107.

Sweet, R.M., Wright, H.T., Janin, J., Chotina, C.H. and Blow, D.M. (1974) Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6 A° resolution. *Biochemistry* **13**: 4212-4228.

Tan-Wilson, A.L., Rightmire, B.R. and Wilson, K.A. (1982) Different rates of metabolism of soybean proteinase inhibitors during germination. *Plant Physiology* **70**: 493-497

Tashiro, M., Asao, T., Hirata, C. and Takahashi, K. (1991) Purification, characterization, and amino acid sequence of foxtail millet trypsin inhibitor III. *Agric. Biol. Chem.* **55**: 419-426.

Tashiro, M., Asao, T., Hirata, C., Takahashi, K. and Kanamori, M. (1990) The complete amino acid sequence of a major trypsin inhibitor from seeds of foxtail millet (*Setaria italica*). *J. Biochem.* **108**: 669-672.

Tashiro, M., Hashino, K., Shiozaki, M., Ibuki, F., and Maki, Z. (1987) The complete amino acid sequence of rice trypsin inhibitor. *J. Biochem.* **102**: 297-306.

Tashiro, M., Kihara, y., Katayama, Y. and Maki, Z. (1989) Purification and characterization of a major trypsin inhibitor II, from foxtail millet. *Agric. Biol. Chem.* **53**: 443-451.

Tashiro, M. and Maki, Z. (1978) Partial purification and some properties of a trypsin inhibitor from rice bran. *Agric. Biol. Chem.* **42**: 1119-1124.

Taylor, B.H., Young, R.J. and Scheuring, C.F. (1993). Induction of a proteinase inhibitor II-class gene by auxin in tomato roots. *Plant Mol. Biol.* **23**: 1005-1014.

Terada, S., Fujimura, S. and Kimoto, E. (1994a) Amino acid sequences of double-headed proteinase inhibitors from the seeds of *Canavalia lineata*. *Biosci. Biotech. Biochem.* **58**: 376-379.

Terada, S., Fujimura, S., Kino, S. and Kimoto, E. (1994b) Purification and characterisation of three proteinase inhibitors from *Canavalia lineata* seeds. *Biosci. Biotech. Biochem.* **58**: 371-375.

Terada, S., Katayama, H., Nodo, K., Fujimura, S., and Kimoto, E. (1994c) Amino acid sequence of Kunitz family subtilisin inhibitors from seeds of *Canavalia lineata*. *J. Biochem.* **115**: 397-404.

Turks, V. and Bode , W. (1991) The cystatins: protein inhibitors of cysteine proteinases. *FEBS Letters* **285**: 213-219.

Udupa, S.L. and Pattabiraman, T.N. (1985) Isolation and characterization of a trypsin/chymotrypsin inhibitor from the Millet *Echinocloa fruneutacea*. *J. Agric. Food Chem.* **33**: 642-646.

Visentin, M., Iory, R., Valdicelli, L. and Palmier, S. (1992) Trypsin inhibitory activity in some rapeseed genotypes. *Phytochemistry* **31**: 3677-3680.

Walker-Simmons, M. and Ryan, C.A. (1977) Immunological identification of proteinase inhibitors I and II in isolated tomato leaf vacuoles. *Plant Physiology* **60**: 61-63.

Walsh, T.A. and Twitchell, W.P. (1991) Two Kunitz-type proteinase inhibitors from potato tubers. *Plant Physiology* **97**: 15-18.

Warchalewski, J.R. (1987) Purification and characteristics of an endogenous alpha-amylase and trypsin inhibitor from wheat seeds. *Die Nahrung* **31**: 1015-1031.

Watson, L. and Dallwitz, M.J. (1992) *The Grass Genera of the World*. CAB International, Wallingford.

Weiel, J. and Hapner, K.D. (1976) Barley proteinase inhibitors: a possible role in grasshopper control. *Phytochemistry* **15**: 1885-1887.

Wen, L., Huang, J.K., Zen, K.C., Johnson, B.H., Muthukrishnan, S., Mackay, V., Manney, T.R., Manney, M. and Reeck. G.R. (1992) Nucleotide sequence of cDNA clone that encodes the maize inhibitor of trypsin and activated Hageman factor. *Plant Mol. Biol.* **18**: 813-814.

Wieczorek, M., Otlewski, J., Cook, J., Parks, K., Leluk, J., Wilimonwska-Peic, A., Polanowski, A., Wilusz, T. and Laskowski, M. (1985) The squash family of serine proteinase inhibitors. Amino acid sequences and association equilibrium constants of inhibitors from squash, summer squash, zucchini, and cucumber seeds. *Biochem. Biophys. Res. Commun.* **126**: 646-652.

Williamson, M.S., Forde, J. and Kreis, M. (1988) Molecular cloning of two isoinhibitor forms of chymotrypsin inhibitor 1 (CI-1) from barley endosperm and their expression in normal and mutant barleys. *Plant Mol. Biol.* **10**: 521-535.

Wilusz, T. Wieczorek, M., Polanowski, A., Denton, A., Cook, J. and Laskowski, M. (1983) Amino acid sequence of two trypsin isoinhibitors, ITD I and ITD III from squash seed (*Cucurbita maxima*). *Hoppe-Seyler's Z. Physiol. Chem.* **364**: 93-95.

Wingate, V.P. and Ryan, C.A. (1991) Uniquely regulated proteinase inhibitor I gene in a wild tomato species. *Plant Physiology* **97**: 496-501.

Wolfson, J.L. and Murdock, L.L. (1987) Suppression of *Colorado* potato beetle growth and development by digestive proteinase inhibitors. *Entomol. Exp. Appl.* **44**: 235-240.

Wong, P.P., Kuo, T. and Ryan, C.A. (1975) Growth-dependent accumulation and utilisation of proteinase inhibitor I in tobacco callus tissues. *Biochem. Biophys. Res. Commun.* **63**: 121-125.

Xavier-Filho, J. (1974) A research note: trypsin inhibitors in sorghum grain. *J. Food Science.* **39**: 422-423.

Xavier-Filho, J. and De Azevedo-Moreira, R (1978) Visualization of proteinase inhibitor in SDS-polyacrylamide gels. *Analytical Biochemistry* **84**: 296-303.

Yoshikawa, M., Iwasaki, T., Fujii, T. and Oogaki, M. (1976) Isolation and some properties of a subtilisin inhibitor from barley. *J. Biochem.* **79**: 765-773.

Young, R.J., Scheuring, C.F., Harris-Haller, L. and Taylor, B.H. (1994) An auxin-inducible proteinase inhibitor gene from tomato. *Plant Physiology* **104**: 811-812.