Proteinase inhibitors from seeds: prospects for their use in plant protection

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Abstract

Seeds constitute a rich source of proteins that can inhibit specific enzyme groups. Thus far, the best characterised are inhibitors of enzymes which play an integral part in the germination process. These include the proteinase inhibitors, and as such they may play a role in the prevention of precocious germination. However, these proteins are members of multi-gene families which also encode iso-inhibitors that can retard the activity of proteinases specifically from mammalian, insect or bacterial (but not plant) origin. A proposed role for this latter group of iso-inhibitors is as seed defensive factors, and it is this group of proteins that is attracting interest from the biotechnology industry.

A large number of proteinase inhibitors have now been identified and evidence for their proposed roles in seeds will be summarised. In addition, their potential as a source of genes that can be transformed into crop plants to enhance field resistance against insects pests will be evaluated.

Additional key words: *insect pest resistance, plant biotechnology, transgenic plants.*

Definitions: Proteinases and Proteinase Inhibitors

In modern nomenclature, proteinases are a subgroup of the peptidases (= proteases), which consists of the endopeptidases (the proteinases; EC 3.4.21-99) and the exopeptidases (EC 3.4.11-19). According to the mechanism of catalysis, proteinases can be grouped further into 'serine', 'cysteine' (thiol), 'aspartic' (acid) and 'metallo'-proteinases which have been assigned the IUB designations of EC 3.4.21-24, respectively.

The serine proteinases are the largest, most widespread and diverse group of these enzymes, and are found in both prokaryotes and eukaryotes. They consist of two superfamilies (the chymotrypsin superfamily and the subtilisin superfamily) and all enzymes in this group are characterised by the presence of two amino acids at the active site that are invariably serine and histidine. The cysteine proteinases are also widespread, and contain cysteine and histidine as the conserved amino acids at the catalytic site. The aspartic proteinases have thus far only been identified in eukaryotes. These enzymes are characterised by a ph optima usually in the range pH 3.5-5.5, and aspartic acid as the catalytically active amino acid. Metalloproteinases are found in both prokaryotes and eukaryotes, and are characterised by zinc (in most cases) being the catalytically active metal (Barrett, 1986; and references therein).

Proteinase inhibitors are themselves proteins, and by definition, can repress the catalytic activity of proteinases (Laskowski and Kato, 1980). In accord with the proteinases, it is appropriate to group proteinase inhibitors into four major classes: serine, cysteine, aspartic and metalloproteinase inhibitors. Within each class, these inhibitors can be grouped further, usually into distinct families, with assignation to a particular family dependent upon amino acid sequence homology. For instance, at least 16 gene families of protein inhibitors of serine proteinases have been identified thus far (Garcia-Olmedo *et al.*, 1987; Bode and Huber, 1992).

In common with proteinases, the inhibitors of these enzymes also have specific amino acids at discrete sites (designated the reactive sites), where hydrolysis by the proteinase occurs. These amino acids occur as a pair, and it is these residues that determine the specificity of the enzyme:inhibitor interaction. However, and unlike natural peptide substrates, the consequence of cleavage at this site by the proteinase is comparatively trivial since

the inhibitor binds essentially irreversibly into the active site of the enzyme (Laskowski *et al.*, 1983; Ryan, 1989; Hubbard *et al.*, 1991).

Three facets commonly distinguish the enzyme / inhibitor association and together these account for an essentially stable and (in kinetic terms) an irreversible complex:

1. Binding of the inhibitor is accompanied by little conformational change of the enzyme,
2. After cleavage of the inhibitor (and unlike polypeptide substrates), the resultant peptides are still held together by disulphide bridges (a common feature of most inhibitors), and
3. The equilibrium constant for the peptide hydrolysis is not very large.

In plant tissues, proteinase inhibitors are widespread, although the majority of these proteins are confined to seeds or other storage organs. In disulphide seeds, for example, trypsin inhibitors can account for up to 6% of total soluble protein (Rackis and Anderson, 1964), and for 10% or more of the total soluble protein in potato tubers (Richardson, 1977). Proteinase inhibitors are more rarely present in aerial plant parts, but do accumulate to substantial levels following wounding. In tomato for instance, these proteins can account for up to 2% or more of the total soluble proteins in wounded leaf tissue (Graham *et al.*, 1985).

The Role of Proteinaceous Inhibitors in Seeds

The presence of proteinaceous inhibitors of proteinases as fully functional entities in seeds was confirmed when the first serine proteinase inhibitor of plant origin was identified in disulphide seeds (Kunitz, 1947). With further characterisation, it became evident that these proteins perform several roles in seeds (Ryan, 1989). Thus far, at least three possible functions have been ascribed universally to these proteins:

1. The prevention of precocious germination caused through the inhibition of any unscheduled proteolytic activity,
2. A putative role, particularly for those inhibitors confined to protein bodies, as seed storage proteins, and
3. Protection of the seed against microbial attack or insect predation.

Prevention of precocious germination

The evidence for the prevention of precocious germination by inhibiting endogenous proteinases is, still, very uncertain. There are reports that show that proteinase inhibitors can retard the activity of endogenous proteinases extracted from seeds. Shain and Mayer (1965) demonstrated that an inhibitor, partially purified from dry lettuce seeds, inhibited 'trypsin-like' enzyme activity in germinating seeds. Likewise Basha and Cherry (1978) observed that the addition of an ungerminated (dry) seed extract from peanut (*Arachis hypogeae* L.) inhibited proteolytic activity in an extract prepared from 10-day old germinating seeds. In a more systematic study, Kirsi and Mikola (1971) identified three groups of proteolytic inhibitors in extracts from resting barley grains. The first group inhibited bovine trypsin, the second inhibited proteinases from *Aspergillus*, while the third inhibited endogenous proteinases. Nevertheless, these experiments, in common with others, used crude seed extracts and so a limitation to accurate conclusions from these studies is the knowledge that *in vivo*, both proteinases and proteinase inhibitors are compartmentalised in seeds.

In this regard, a particularly noteworthy study by Baumgartner and Chrispeels demonstrated that dry mung bean seeds contain a low molecular weight inhibitor of the major endopeptidase induced in seeds during germination (Baumgartner and Chrispeels, 1976; Chrispeels and Baumgartner, 1978). As predicted, the concentration of the inhibitor declines rapidly during germination (although at a faster rate than the observed increase in proteinase activity), but critically, the inhibitor was shown to be cytosolic in location, while the proteinase activity was associated with the protein bodies. The authors suggest that the function of the inhibitor is to protect the cytoplasmic contents from proteinases in the event of accidental rupture of the protein bodies during storage protein degradation. These experiments illustrate the need to characterise accurately the endogenous proteinases that are involved in the germination process, the affinity of endogenous inhibitors for these proteinases and the subcellular location of both types of proteins.

Seed storage proteins

The second putative role for proteinase inhibitors is as seed storage proteins, particularly in legume seeds in which these proteins are known to be located in protein bodies. In addition, most inhibitors contain a high proportion of the amino acid cysteine, and as such are proposed to represent a storage form of sulphur for the germinating seed. However, there is, as yet, little

compelling evidence for a role of these inhibitors as seed storage proteins. For those seeds that have been examined, some modification to these inhibitors does occur during germination. However, these alterations are, in the main, precise proteinase-induced events such that the inhibitor does not lose activity, suggesting that these proteins maintain some functional capacity during germination.

As an illustration, in germinating mung beans there is a three-day lag period after the start of imbibition before significant degradation of storage protein is observed (Lorensen *et al.*, 1981). During this lag period, an endogenous trypsin inhibitor becomes modified, but the actual concentration of the protein remains essentially constant. The data suggests a specific pathway for the initial stages of modification resulting in distinct forms of the inhibitor. The major species that exists in the quiescent dry seed is a trypsin inhibitor with 80 amino acid residues in a single polypeptide chain. The first modification involves the loss of four amino acids at the carboxyl terminal, followed by the loss of two further amino acids from the carboxy terminal and eight amino acids from the amino terminal as a second modification. Each of these modified forms can be isolated as an identifiable entity that still retains inhibitory activity (Wilson and Chen, 1983).

Such modifications to proteinase inhibitors have also been observed in other species, including to one of the major trypsin inhibitors in disulphide seeds (the Kunitz inhibitor). Here, modification appears to result from the loss of approximately 10 amino acids from the carboxyl terminus of the native protein. Beyond this point, the inhibitor does not appear to be significantly degraded during the germination process and retains its activity (Tan-Wilson *et al.*, 1982).

Protective functions

In terms of the third assigned role for proteinase inhibitors in seeds, there is now little doubt that these proteins can inhibit proteolytic enzymes derived from insect digestive tracts, particularly the serine proteinases. This mode of protection against insect predation is thought to have evolved as part of a multifarious mechanism of defence used by plants ranging from purely morphological and structural adaptations to the biosynthesis of complex secondary compounds. The experimental evidence that supports this particular role comes from the interpretation of two distinct aspects of investigation.

In the first, Green and Ryan (1972) discovered that there was a rapid induction in the synthesis of proteinase inhibitors in potato and tomato plants when injury occurred. The inhibitor content of leaves and stems was low in undamaged plants but increased rapidly throughout the plant's tissues when Colorado beetle fed on individual leaves. The authors concluded that a rise in the levels of inhibitor after injury may be part of the plants' defence against insect attack. Subsequently, a polygalacturonide 'wounding signal' has been identified that can induce the expression of these inhibitors in remote plant parts following localised insect damage (Ryan *et al.*, 1985).

For the second, Lipke and co-workers observed that a protein fraction from disulphide seeds inhibits, *in vitro*, proteolytic activity that has been isolated from larvae of *Tribolium confusum* (Lipke *et al.*, 1954). Following this initial observation, enzymes from the digestive tract of several insect larvae have been shown to be inhibited by the Kunitz trypsin inhibitor from disulphide seeds. These include the midgut proteinases of the meal beetle, *Tenebrio molitor* (Applebaum *et al.*, 1964) and the trypsin-like enzyme of the tobacco hornworm, *Manduca sexta* (Miller *et al.*, 1974).

In sum then, it appears that insect attack can induce the biosynthesis of proteinase inhibitors in plants and that, as part of their spectrum of activity, these proteins can retard proteolytic activity extracted from the digestive tract of insect pests.

Prospects for the Use of Inhibitors as Plant Protection Factors

The potential use of proteinase inhibitors to protect crop plants against insect damage was given further impetus from many studies in which these proteins were added to insect diets and the development of the feeding insect larvae observed. For instance, Steffens and colleagues (Steffens *et al.*, 1978) included two purified inhibitors (the Kunitz trypsin inhibitor from disulphide and a trypsin inhibitor from maize) in diets of the young corn borer larvae, *Ostrinia nubilalis*, and observed that the more potent of the two inhibitors (the Kunitz), had the greater effect in retarding growth and metamorphosis of the larvae. In a more recent study, Broadway and Duffy (1986) included the Kunitz trypsin inhibitor (from disulphide) and potato proteinase inhibitor II in diet fed to larvae of *Heliothus zea* and *Spodoptera exiqua* and observed a marked reduction in the growth and development of feeding insects.

A more certain link between the accumulation of proteinase inhibitors and resistance of the plant to insect attack was determined by Gatehouse and colleagues (Gatehouse *et al.*, 1979; Gatehouse and Boulter, 1983).

These workers investigated the biochemical basis for resistance of seeds of the cowpea, *Vigna unguiculata* (L.) (Walp.), to the bruchid beetle, *Callosobruchus maculatus* (F.). Some five thousand varieties had previously been screened and one, TVu 2027, showed a significant reduction in damaged caused by larvae of *C. maculatus*. Seeds were tested from this variety for the presence of various anti-metabolic compounds, and a very high level of trypsin inhibition activity was discovered (about two-fold higher than for other varieties). The trypsin inhibitor was purified from cowpea by affinity chromatography on trypsin-Sepharose and confirmed to be the toxic determinant in insect feeding trials when artificial diet was supplemented with this inhibitor.

Nevertheless, it was not until the development of techniques for the direct introduction of foreign genes into higher plants (Hernalsteens *et al.*, 1980; Herrera-Estrella *et al.*, 1983) that a mechanism for the use of proteinase inhibitors as crop protection agents could be designed - that is, the direct introduction of genes encoding these proteins into target plants.

In 1987, Hilder and co-workers introduced the gene encoding a cowpea trypsin inhibitor into tobacco, confirmed that it was expressed, and demonstrated that the presence of the inhibitor retarded the growth rate of feeding newly emerged larvae of *Heliothus virescens* (Hilder *et al.*, 1987). A direct correlation was observed between the accumulation of the inhibitor in leaf tissue and the incidence of insect survival.

More recently, Johnson and colleagues (Johnson *et al.*, 1990) transformed tobacco with either one of two genes encoding trypsin inhibitors from the multi-gene proteinase inhibitor II families of tomato and potato, or a gene encoding a chymotrypsin inhibitor (from the tomato proteinase inhibitor I gene family). They observed that the accumulation of these trypsin inhibitors correlated with significantly depressed growth rates of feeding *Manduca sexta* larvae, while no depression of growth rate was observed for larvae fed on transgenic tobacco accumulating the tomato proteinase inhibitor I protein. The authors concluded that inhibitory activity against trypsin, but not chymotrypsin, was mainly responsible for the depressed larval growth rates they observed.

Current research in New Zealand

Studies from this laboratory have shown that for some insects, chymotrypsin inhibitors can be effective as feeding deterrents in transgenic plants (McManus *et al.*, 1993a). Tobacco has been transformed with a member of the potato proteinase inhibitor II gene family (this gene encodes for an iso-inhibitor with predominantly chymotrypsin, rather than trypsin inhibitory activity). The gene was introduced as a transcriptional fusion, under the control of a promoter cloned from the genome of the cauliflower mosaic virus (designated the 35S promoter), such that this fusion confers a satisfactory level of gene expression in all plant parts. Accumulation of potato proteinase inhibitor II protein in leaf tissue was determined using a specific antibody (a survey of the range of proteinase levels obtained from different transgenic plants is given in Table 1). Functional activity of the inhibitor was confirmed using extracts from transgenic leaf tissue in a chymotrypsin enzyme assay. As expected, increasing the amount of leaf protein added to the assay, decreased the activity of the enzyme correspondingly. No inhibition of activity was observed with extracts from control (non-transgenic) tissue (Fig. 1). Results from insect feeding trials determined that the accumulation of the proteinase inhibitor in tobacco was effective in retarding larval development of *Chrysodeixis eriosoma*, a New Zealand pest of the Solanaceae. Accumulation of the inhibitor was, however, ineffective at hindering the development of a closely related insect pest, *Thysanoplasia orichalcoa* when fed transgenic tobacco (data from typical insect growth trials are plotted as Figure 2).

The observation that the growth of closely related insect species can be affected quite differently by the same proteinase inhibitor is significant, because it warns against the exclusive reliance on a single proteinase inhibitor to provide enhanced resistance to a wide variety of insects. Instead, dual approaches, perhaps involving two proteinase inhibitors, or a proteinase inhibitor in concert with another determinant that utilises a different

Table 1. Quantification of the accumulation of potato proteinase inhibitor II in transgenic tobacco leaves using an enzyme-linked immunosorbent assay (ELISA).

Plant Reference	Potato Proteinase Inhibitor II (percentage of total leaf protein)
6857-1	0.080
6857-4	0.004
6857-6	0.120
6857-7	0.015
6857-12	0.088
6857-13	0.125
Control	-
Control	-

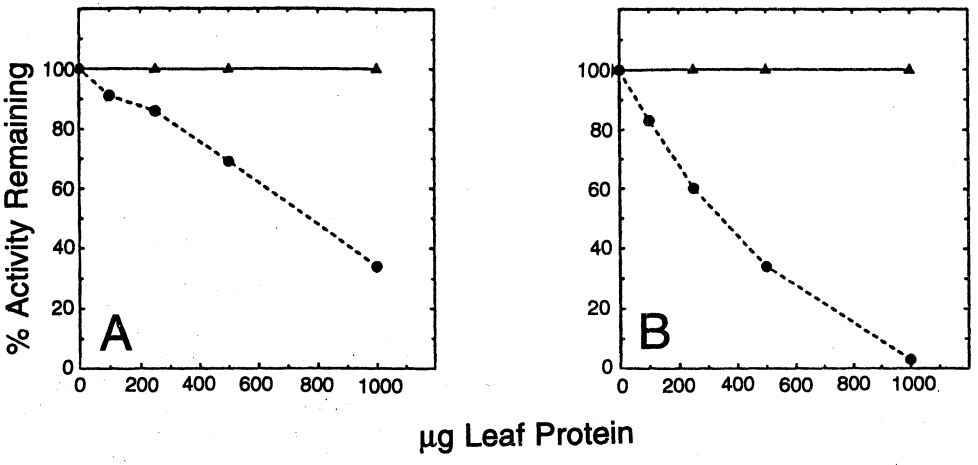

Figure 1. Inhibition of chymotrypsin activity by extracts from either transgenic (-•-), or control (-▲-) tobacco plants. A = Plant 6857-1; B = Plant 6857-13.

mechanism to provide insect toxicity should be employed. Notwithstanding this, the early success of the use of these proteins as insect pest resistance factors in transgenic plants augurs well for the inclusion of proteinase inhibitors in the development of such strategies.

For New Zealand agriculture, the transformation of pasture plants with proteinase inhibitors should enhance the field resistance of the transformants against major pasture pests such as grass grub (*Costelytra zealandica*), and this approach is now well advanced in this laboratory. The rationale is to first identify the major digestive proteinases in the insect pest and then select an appropriately potent inhibitor. This approach has been successful in formulating strategies to combat the Colorado potato beetle, *Leptinotarsa decemlineata* (Wolfson and Murdoch, 1987). The major digestive proteinase was identified as a cysteine proteinase (rather than a serine proteinase) and in feeding trials, supplementation of artificial diet with E-64 (a synthetic cysteine proteinase inhibitor) suppressed the growth and development of feeding beetle larvae (serine proteinase inhibitors were ineffective). Genes encoding proteinaceous inhibitors of cysteine proteinases have now been cloned and so this strategy can be extended to transgenic plants (Turk and Bode, 1991).

Christeller and colleagues have surveyed the spectrum of proteinases in the digestive tract of the grass grub and identified the serine proteinase, trypsin as the major enzyme, augmented with smaller amounts of chymotrypsin activity (Christeller *et al.*, 1989). These workers have also undertaken a comprehensive screening of inhibitors of grass grub trypsin and found many to be effective at retarding the activity of the enzyme (Christeller and Shaw, 1989).

Based on these studies, genes encoding a chymotrypsin inhibitor (potato proteinase inhibitor II, Keil *et al.*, 1986) and a Kunitz trypsin inhibitor (from disulphide, Jofuku and Goldberg, 1989) are being introduced simultaneously into the forage legume white clover (*Trifolium repens*). We have evidence that both types of inhibitors are effective, *in vitro*, against grass grub trypsin and chymotrypsin (McManus *et al.*, 1993b). Our expectation is that this dual inhibitor approach will confer a degree of field resistance to the pasture plant against grass grub. This is important, since conventional plant breeding approaches have, thus far, failed to produce white clover cultivars that are resistant to grass grub (Dymock *et al.*, 1989).

It is the realisation that agronomically-desirable traits can be introduced directly into plants (particularly where conventional plant breeding has been unsuccessful) that will guarantee the further development and continued success of this experimental approach. Proteinase inhibitors will have a role to play as part of the overall composite of pest resistance factors available for

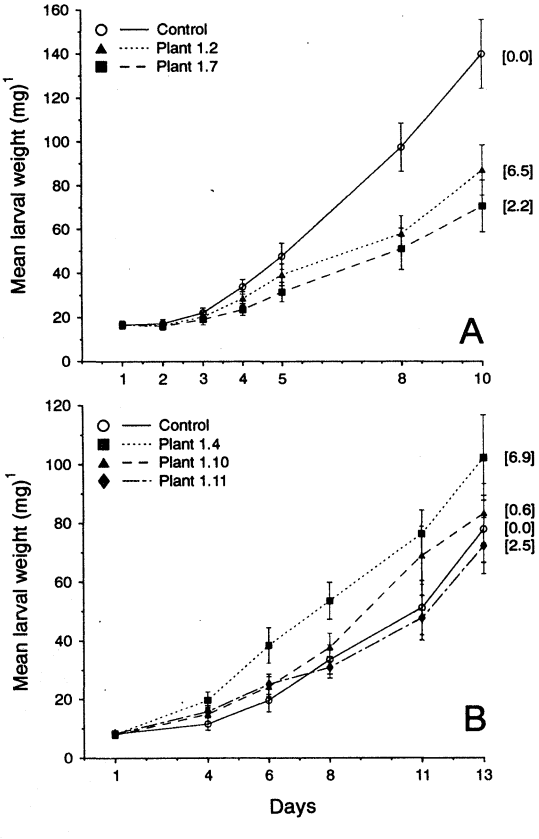

Figure 2. Insect feeding experiments. a) Mean weights of larvae of *Chrysodeixis enosoma* fed leaf tissue from either the progeny of proteinase inhibitor II (PPi II) transformed plant, 6857-1 (1.2, 1.7) or from a control (non-transgenic) plant. b) Mean weights of larvae of *Thysanoplusia orichalcoa* fed leaf tissue from either the progeny of proteinase inhibitor II (PPiII) transformed plant, 6857-1 (1.4, 1.10, 1.11) or from a control (non-transgenic) plant. (The levels of PPiII accumulation in each plant, as determined by ELISA are given in brackets as mg PPiII/µg leaf protein; from McManus *et al.*, 1993a).

introduction into plants. More research into proteinase inhibitors therefore, particularly their role in seeds, will enhance further their potential effectiveness to biotechnologists.

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