The functions of proteinase inhibitors in seeds

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Abstract

Proteinase inhibitors comprise a significant component of the protein content of many seeds and at least three functions have been ascribed to this group of proteins. They have been proposed to function as (1) protectants against seed predators, (2) as storage proteins, and (3) as regulators of endogenous protease activity, particularly in the dry seed. The evidence which supports each proposed function is reviewed, with greatest emphasis on whether these proteins do function as regulators of endogenous proteins in seeds. More recently, the use of more sensitive detection methods has revealed a further tier of proteinase inhibitors which accumulate at very much lower concentrations than those inhibitors normally described from seeds. Using apple seeds as an example, the inhibitory activity of these proteins is described and some speculation as to their likely roles in seeds is included.

Additional key words: *cystatins, cysteine proteases, insect pest resistance, serine proteases*

Introduction

Proteinase inhibitors are (typically) lower molecular weight proteins which repress the catalytic activity of proteinases (Laskowski and Kato, 1980). In plants, they are commonly found constitutively in seeds and storage organs, but are also induced in other plant tissues in response to wounding (Garcia-Olmedo *et al.*, 1987; Richardson, 1987). More recently, proteinase inhibitors have also been shown to be induced in response to water stress (Downing *et al.*, 1992) and during organ senescence and abscission in higher plants (Buchanan-Woolasten, 1997; Coupe *et al.*, 1997).

The classification of proteinase inhibitors is determined by the mechanistic class of proteinase repressed. Using the nomenclature of Barett (1998), proteinases are a subgroup of the proteases (= peptidases) which comprises the exopeptidases (EC 3.4.11-19) and the endoproteases (the proteinases; EC 3.4.21-99). According to their catalytic mechanism, proteinases can be divided further into the serine, cysteine (thiol), aspartic (acid) and metalloproteinases, and with the exception of the aspartic proteases, members of each group have been identified in both eukaryotes and prokaryotes. In addition to which class of proteinase they inhibit, plant proteinase inhibitors can also be subgrouped further by virtue of sequence homology and have now been classified into a number of families (Table 1). The largest number of inhibitor families are active against the serine proteinases, which may reflect their abundance in plants, but also that they were the first characterised and are probably the most studied (Garcia-Olmedo *et al.*, 1987). Inhibitors against the other proteinases are also present in plants, with the phytocystatins perhaps the fastest growing group in terms of the numbers of new inhibitors being characterised. It is most likely that this family will be divided further in the future.

With the inclusion of aspartate proteinase inhibitors, examples of all inhibitors of proteinase classes are found in seeds, although not representatives of all families. Again, families with specificity towards the serine proteinases are the best characterised in seeds, particularly the Kunitz and Bowman-Birk families expressed constitutively in seeds of leguminous species

Table 1. Plant proteinase inhibitor families[1].

Serine proteinase inhibitors	
	Soybean (Kunitz) trypsin inhibitor family
	Bowman-Birk family
	Potato inhibitor I family
	Potato inhibitor II family
	Barley trypsin inhibitor family
	Squash inhibitor family
	Ragi I-2/maize trypsin inhibitor family
	Serpin family
Cysteine proteases	
	Phytocystatins
Metallo-proteinase inhibitors	
Aspartic protease inhibitors	

[1]Modified from Koiwa *et al.* (1997).

and the trypsin/α-amylase family (the Ragi I-2/maize bifunctional inhibitor in Table 1) expressed constitutively in cereal grains and in dry seeds of the cultivated grasses (Garcia-Olmedo *et al.*, 1987; Tasneem *et al.*, 1994; 1996). The other major serine proteinase families in plants are the potato proteinase inhibitor I and II families. These are found predominantly in solanaceous species, either expressed constitutively in storage organs (potato tubers, fruit pericarp tissue) or are wound-inducible in other plant tissues, for example in leaf tissue (Pearce *et al.*, 1993, McManus *et al.*, 1994a). However, a Bowman-Birk inhibitor has been shown to be induced by wounding in leaves of lucerne (Brown *et al.*, 1985), the Kunitz trypsin inhibitor has been identified in the phloem of stem tissue of winged bean (Habu *et al.*, 1996) and an inhibitor with identity at the amino acid level to members of the Potato inhibitor I family has been identified in seeds of amaranthus (Valdes-Rodriguez *et al.*, 1993).

Cysteine proteinase inhibitors were first identified in pineapple (Reddy *et al.*, 1975) and in terms of those of seed origin have now been characterised in rice grains (Abe *et al.*, 1987), endosperm tissue of corn (Abe and Whitaker, 1988), in cowpea (Fernandes *et al.*, 1993), sunflower (Kouzuma *et al.*, 1996), soybean (Misaka *et al.*, 1996), bean (Brzin *et al.*, 1998; Santino *et al.*, 1998) and in apple (Ryan *et al.*, 1998).

It is clear, therefore, that with more investigation of proteinase inhibitors in seeds, many more representatives of each family will be identified, as will representatives from families previously considered to be absent from seeds. However, the identification and characterisation of proteinase inhibitors in seeds is linked intimately to the method of study. Inhibitor proteins are detected by virtue of inhibition of proteinases and it is the level of sensitivity of the proteinase assay that determines whether inhibitors in lower abundance can be discerned. More recently, the use of fluorogenic-linked substrates has meant that very sensitive detection of seed proteases is now possible and so, in principle, proteinase inhibitors in very low abundance can be detected. The first inhibitors identified in seeds were the abundant serine proteinase inhibitors where the levels of these proteins suggests that they function as seed protectants/storage proteins. More recently, the cystatins, which appear to be present in significantly lower concentrations are being identified using the more sensitive protease inhibitor assays.

The literature pertaining to the characterisation of proteinase inhibitors in seeds is vast and for this review, we propose to consider only the functions of proteinase inhibitors in seeds. This is, in part, because fewer reviews have dealt with this aspect, but also because the use of more sensitive proteinase substrates has revealed a tier of proteinase inhibitors that have only been identified through the use of these sensitive substrates. These groups of inhibitors, because of their abundance, are more likely to be involved in the regulation of endogenous proteases. Our recent studies on cystatins from apple seeds, identified using fluorogenic substrates, will be reviewed in this context.

Protection Against Seed Predation

The proposal that proteinase inhibitors from seeds may act as protectants arose from early experiments which demonstrated that protein extracts from soybean seeds inhibited growth and digestive proteolytc activity, *in vitro*, of feeding larvae of *Tribolium confusum* (Lipke *et al.*, 1954), and that the purified Bowman-Birk trypsin inhibitor, when added to an artificial diet, retarded the growth of larvae of the closely related *Tribolium castaneum* (Birk and Applebaum, 1960). These early experiments, which showed that proteinase inhibitors from seeds could retard insect growth *in vitro*, were supported by the observation of Green and Ryan (1972) that wounding of leaf tissue of tomato induced the production of proteinase inhibitor I, and that when wounded leaf material was fed to insect larvae, then growth was retarded (Broadway *et al.*, 1986). In parallel to these wounding experiments, Gatehouse and colleagues screened seed from five thousand varieties of cowpea, *Vigna unguiculata* L., for resistance to the bruchid beetle, *Callosobruchus maculatus* (F.) and showed that one variety, Tu 2027, demonstrated a significant reduction in damage caused by feeding beetle larvae (Gatehouse *et al.*, 1979). Further investigation of this variety revealed a significantly higher level of trypsin inhibition in the seeds when compared with other varieties, and when the purified trypsin inhibitor was fed to *C. maculatus* larvae, inhibition of growth was observed (Gatehouse and Boulter, 1983). While the occurrence of trypsin inhibition has been challenged as the primary determinant of resistance to the bruchid beetle in Tu 2027 (Xavier-Filho *et al.*, 1989), the use of proteinase inhibitors from seeds has now had a considerable impact in the deployment of these proteins as insect resistance factors in transgenic plants (Hilder *et al.*, 1987; McManus *et al.*, 1994b; Xu *et al.*, 1996; Gatehouse *et al.*, 1997; Confalonieri *et al.*, 1998; De Leo *et al.*, 1998; Gatehouse *et al.*, 1999; McManus *et al.*, 1999).

Proteinase inhibitors from seeds have also been advocated as protective agents against microbial

pathogens. For example, inhibitors from seeds of kidney bean have been shown to repress protease activity *in vitro* of fungal proteinases (e.g., Mosolov *et al.*, 1979) and an inhibitor from buckwheat seeds is effective at retarding the mycelial growth and germination of spores of two species of filamentous fungi (Dunaevsky *et al.*, 1997). Further, more detailed characterisation of the properties of some proteinase inhibitors from seeds have revealed that particularly abundant proteins are inhibitory to subtilisin, a major chymotrypsin subclass of the serine proteinases found in microorganisms. For example, barley grains contain two such inhibitors, designated CI-1 and CI-2 (Boisen *et al.*, 1981).

In common with the serine protease inhibitors, the phytocystatins have been shown to be active against cysteine proteases in insect digestive tracts *in vitro* (Hines *et al.*, 1991; Liang *et al.*, 1993) and have also been used successfully against insect pests in transgenic plants (Leple *et al.*, 1995; Irie *et al.*, 1996).

However, before leaving the discussion of proteinase inhibitors as seed protectants, it should be noted that apart from the study on bruchid beetle resistance in cowpea (Gatehouse *et al.*, 1983), there is no direct evidence that this is the function of these proteins in nature. Surveys of the occurrence of proteinase inhibitors in a wide range of plant species have been undertaken (Janzen *et al.*, 1986), but convincing correlative data between the occurrence of specific inhibitors and the feeding of insect pests is still lacking.

Seed Storage Proteins

The proposed role for proteinase inhibitors as storage proteins was suggested first by Pusztai (1972) who showed that during the germination of kidney bean, the period of maximum proteinase inhibitor content (7 - 8 days after germination) coincided with the period of maximum proteolysis. Pusztai proposed that these proteins were not regulators of endogenous trypsin, but may serve as a source of important sulphur-containing amino acids for the germinating seed.

The two seed proteinase inhibitors which have been used most widely for transgenic plant studies to confer insect resistance (the cowpea trypsin inhibitor and the soybean Kunitz inhibitor) are well represented in terms of proportion of total seed protein (Rackis and Anderson, 1964; Gatehouse *et al.*, 1979). The demonstrated potency of these proteins at retarding insect proteinases is good evidence that seed proteinase inhibitors act as protectant proteins. However, their abundance has also been interpreted by many researchers as a function as seed storage proteins, a role which is not mutually exclusive to that of seed protectant. For example, the major storage albumin from *Theobroma cacao* seeds with homology with the Kunitz family of protease inhibitors comprises 25-30 % of the total seed protein and is proposed to have a dual protection/storage function (Spencer and Hodge, 1991). It would appear advantageous, therefore, for germinating seedlings to remobilise the significant nitrogen reserves contained in proteinase inhibitors after the need for protection has lapsed.

The assessment of proteinase inhibitors as storage proteins is complicated by the observation that in seeds of many species, germination is accompanied by the appearance of new forms of inhibitors (see for example, Ambeker *et al.*, 1996; Harsulkar *et al.*, 1997; Sreerama and Gowda, 1998). This is not unexpected since these proteins will have important functions in the developing vegetative tissues of the germinating seedling. However, characterisation of these newly formed inhibitors in seeds at the protein sequence level has shown that they can arise from modification of existing proteinase inhibitors in the dry seed.

In germinating mungbean, the level of the major trypsin inhibitor (designated as MBTI-F) is observed to decrease as it is converted firstly to form E, then C and finally A, with these final forms (A and C) the dominant inhibitor proteins in germinated seeds at 96 h (Lorensen *et al.*, 1981). Amino acid sequencing of MBTI-F subsequently revealed that it was a typical Bowman-Birk serine proteinase inhibitor and that the conversion of F to E involved the loss of a C-terminal tetrapeptide, while a further two amino acid residues are removed from MBTI-E to form C (Wilson and Chen, 1983). The proteinase involved in the conversion of MBTI-F to E has been characterised and shown to be a cysteine proteinase (Wilson and Tan-Wilson, 1987).

The two major species in soybean seeds, the Bowman-Birk and Kunitz inhibitors have also been shown to undergo proteinase-mediated modification during germination (Tan-Wilson *et al.*, 1982). The major form of Bowman-Birk inhibitor in the cotyledons of dry seeds is designated as BB-E which then decreases in content during germination to be replaced by BB-D as the major form in cotyledons at 6 days post-germination. Comparison of amino acid sequences reveals that BB-D is converted to BB-E by the removal of two amino acids (Madden *et al.*, 1985). Likewise, the Ti^a variant of the Kunitz inhibitor is converted to the Ti^a_m form by the removal of five carboxy terminal amino acids and the Ti^b variant is converted to the Ti^b_m form by the removal of a carboxy terminal decapeptide (Hartl *et al.*, 1986). The protease which initiates the conversion of the Ti^a variant to Ti^a_m has been characterised and shown to be a cysteine

protease, the activity of which is induced post-imbibition (Papstoitsis and Wilson, 1991). The same protease is also proposed to participate in the conversion of the Bowman-Birk inhibitor BB-E to BB-D.

Using a genetic approach in pea seeds, Domoney and colleagues have identified two genes in the trypsin family with homology to the Bowman-Birk family, and proposed that the multiple forms of trypsin inhibitors observed in pea seeds using protein purification techniques (Domoney *et al.*, 1993) have arisen by posttranslational modification, particularly by truncation of carboxyterminal amino acids (Domoney *et al.*, 1995). Interestingly, the truncated forms of the inhibitor show a greater affinity for their target proteases suggesting that these modifications are an integral part of the germination process. Truncation of proteinase inhibitors to release active forms has been shown to occur in other families. For example the full-length potato proteinase II inhibitor (12,000 Da) has been shown to be posttranslationally truncated to produce a inhibitory peptide of 5400 Da (McManus *et al.*, 1994c).

The appearance of new forms of inhibitors during germination which are derivatives from those stored in the dry seed (and may be more active) argues against a primary role for these proteins as storage proteins. However, given that these modified inhibitors do eventually disappear supports the notion for a secondary role in which the protein nitrogen is utilised by the growing seedling.

Regulation of Seed Proteases

The evidence that seed proteinase inhibitors may be active against endogenous proteinases came initially from studies on the interaction of trypsin-like proteases and proteinase inhibitors in lettuce seeds (Shain and Mayer, 1965). A partially purified inhibitor preparation was active at retarding a trypsin-like activity induced post-imbibition, but was not active against trypsin-like activity in the dry seed. Kirsi and Mikola (1971) characterised three classes of proteinase inhibition in crude extracts from dry seeds of barley: (i) inhibition of trypsin, (ii) inhibition of an alkaline protease activity from *Aspergillus oryzae*, and (iii) inhibition of endogenous caseinase activity, with highest inhibitory activity of this endogenous activity in the embryo when compared with the endosperm. Horiguchi and Kitagishi (1971) partially purified a trypsin inhibitor from the embryo of rice seed and demonstrated that it could inhibit a partially purified caseinase activity from rice seed. In corn endosperm, a partially purified trypsin inhibitor has been shown to be active against an endogenous activity that catalysed the synthetic substrate N-benzoyl-$_{DL}$-arginine p-nitroanilide (BApNA) (Reed and Penner, 1978), while in pine seeds, crude extracts of whole seeds have been demonstrated to inhibit endogenous haemoglobin-hydrolysing and caseinase activity (Salmia and Mikola, 1980). A proteinase inhibitor has been purified from wheat kernels and found to be effective against a partially purified endogenous trypsin-like activity (Poerio *et al.*, 1989).

Such experimental approaches in which the purified or partially purified inhibitor are tested for retardation of endogenous proteinase activity in essentially crude extracts supports the contention that a function for some of these inhibitors is to regulate endogenous proteinase activity. However, studies with pea seeds have shown that crude trypsin inhibitor preparations were ineffective at retarding azoglobulytic activity in cotyledon extracts (Hobday *et al.*, 1973). This observation was confirmed with studies in pea using either partially purified or two wholly purified inhibitors which were ineffective against a partially purified endogenous protease from whole seed extracts (Domoney *et al.*, 1993).

The previous studies all used substrates that were either non-specifc (for example caseinolytic) or general to the level of a family (trypsin-like proteinases recognise the synthetic substrate BApNA) suggesting that it is likely that more than one proteinase was operating in the assay. To gain more definitive evidence for a role for these proteins in regulating the activity of endogenous proteinases, purified inhibitors and their respective proteinases have been used. Gennis and Cantor (1976) purified two double-headed serine proteinase (Bowman-Birk) inhibitors and an endogenous seed protease from black-eyed peas and demonstrated the formation of an inhibitor:protease complex. Interestingly, the seed protease was unstable if dissociated from the inhibitor suggesting that the role of the inhibitor was not to protect stored protein reserves from unscheduled protease action but to regulate the activity of the protease - an activity which may include degradation of stored reserves during germination.

Using a different approach, Morita *et al.* (1996) purified a serine protease from dry seeds of soybean and determined that activity could be repressed by both the Kunitz and Bowman-Birk inhibitors. This is a significant observation because both the Bowman-Birk inhibitor and the Kunitz inhibitors are major proteins in dry seeds and both have been shown to be effective against insect proteases suggesting their function is one of plant protection (Johnston *et al.*, 1993; McManus and Burgess, 1995). The demonstration that they also can inhibit a specific soybean protease underlines the value of using purified inhibitors and proteases in such functional

studies. However, the inhibition of this protease by these major occurring inhibitors may not have any physiological relevance. An important further component to the determination of such functional relevance is the subcellular localisation of both protease and inhibitor.

Many studies have sought to localise proteinase inhibitors in plants and then to correlate this with the localisation of the major storage protein degrading proteinases. One approach has been to isolate protein bodies and to then probe for the occurrence of proteinase inhibitors. This approach has been used to localise the inhibitory activity against the major storage-degrading endopeptidase in mungbean cotyledons, in which most of the trypsin inhibitory activity was localised in the cytoplasm and not the protein bodies containing the major endopeptidase (Baumgartner and Chrispeels, 1976). These workers concluded that the main function of the inhibitor is to protect the inhibitor from accidental rupturing of the protease-containing protein bodies. In Scots pine seeds, the major inhibitor is proposed to protect cellular structures, particularly in the embryo, within the high proteinase environment during germination and not to regulate the breakdown of storage proteins (Salmia, 1980). Alternatively, Elpidina *et al.* (1991) localised a metalloproteinase which is associated with the proteolysis of storage protein and its inhibitor to the protein bodies of dry buckwheat seeds.

However, it has been claimed that the use of isolated protein bodies gives conflicting data since a major drawback of the technique is the successful isolation of these subcellular organelles (Hobday *et al.*, 1973; Wilson and Wilson, 1987). Instead, immunological techniques have been used to localise proteinase inhibitors with more certainty. Chrispeels and Baumgartner (1978), used the technique to localise a trypsin inhibitor in the cytosol (although this inhibitor is distinct from that reported by Baumgartner and Chrispeels (1976) and is not active against the major endopeptidase in the protein body). In the dry seed of soybean, the Kunitz inhibitor has been localised primarily in the protein bodies of the cotyledons, but also in the embryonic axis using immunogold staining and electron microscopy (Horisberger and Tacchini-Vonlanthen, 1983). Some Kunitz inhibitor was also localised in the cytoplasm of the cotyledon and embryonic axis, and a significant proportion was also recognised in the cell wall of the cotyledon. This cell wall localisation was confirmed using monoclonal antibodies and western analysis (McManus *et al.*, 1995). The major chymotrypsin (subtilisin) inhibitor from barley, CI-2, has been localised to the protein bodies in the endosperm (Rasmussen *et al.*, 1990). Clearly, the use of purified proteinases and matching inhibitors in combination with the subcellular localisation of these proteins will aid the elucidation of the role of inhibitors in regulating the activity of endogenous proteinases.

These localisation studies, predominantly, involve serine proteinase inhibitors and many of these proteins are quite abundant in seeds. More recently, cysteine proteinase inhibitors (phytocystatins) are also becoming better characterised in seeds. In an examination of seeds from a wide range of plant species, Valevski *et al.* (1991) determined that the abundance serine proteinase inhibitors ranged widely (0 to ca. 100,000 units/g), while the range of the cysteine proteinase inhibitors was more restricted (1.7 - 31.7 units/g). As well as these differences in range, in general, cysteine proteinases occur in lower abundance. In a more detailed examination of cowpea, Valevski *et al.* (1991) determined that the cysteine proteinase inhibitors were present in much less abundance when compared with the trypsin inhibitors, suggesting that the cysteine proteinase inhibitors were involved in the regulation of physiological/metabolic processes while the trypsin inhibitors acted as seed protectants. Likewise, Santino *et al.* (1998) purified a cysteine inhibitor from mature seeds of bean and determined that it was present at 0.1 % of the total protein content. Again, the authors proposed that this inhibitor was concerned with the regulation of endogenous cysteine proteinase activity.

Perhaps the best characterised cysteine proteinase inhibitors are those characterised from seeds of rice and maize. A papain inhibitor was first identified in rice seeds (Abe and Arai, 1985) and purified and characterised further by Abe *et al.* (1987a). Significant progress towards characterising the function of the inhibitor has come with cloning the gene that codes for the inhibitor, and expression studies have shown that the inhibitor is expressed maximally 2 weeks after flowering and then gradually decreases to undetectable levels at 10 weeks (Abe *et al.*, 1987b). The expression is coordinated with that of glutelin accumulation and the authors propose that the inhibitor may be concerned with protection against unscheduled storage protein hydrolysis. A second cysteine protease gene has been cloned from rice, the expression of which is highest in the dry seed (Kondo *et al.*, 1990). The protein product inhibits papain, but also demonstrates differences in the profile of other cysteine proteinases which are inhibited suggesting that different endogenous proteinases are regulated by the two inhibitors in rice seeds.

In maize endosperm, Abe *et al.* (1980) isolated an inhibitor (designated CI-4a) which is active against the major thiol protease responsible for the degradation of

zein. Again, by analysis of the timing of inhibitory activity and protease activity post-imbibition, the authors concluded that the inhibitor is important in regulating protease activity in the dry seed. The inhibitor has now been purified from dry maize kernels and its biochemical properties characterised (Abe and Whitaker, 1988; Abe and Arai, 1991). More recently a cDNA encoding a cysteine proteinase from maize endosperm tissue was cloned and expression studies demonstrated that it was expressed maximally 2 weeks after flowering and then continuously during the following maturation stage (Abe *et al.*, 1992). The authors concluded that particularly with respect to the pattern of storage protein deposition, the function of the inhibitor may be to regulate the activity of endogenous cysteine proteinases. Subsequently, the cDNA was expressed in *E. coli* and the inhibitory properties of the protein product characterised as well as the localisation of the inhibitor protein undertaken in the seed (Abe *et al.*, 1994). Kinetic analysis of the protein product revealed that the cDNA did not code for the protein purified previously from maize (Abe and Whitaker, 1988; Abe and Arai, 1991) which was shown to inhibit the zein-degrading protease activity in maize (Abe *et al.*, 1980), and so the actual target protease still needs to be characterised before some assignation of function can be made. However, the subcellular location of the inhibitor revealed that it was present mostly in the aleurone layer with some in the embryo suggesting that the inhibitor may function in regulating the endogenous storage protein-degrading proteases which are also located in these tissues.

Botella *et al.* (1996) identified 3 cDNA clones encoding distinct cysteine proteinase inhibitors from a soybean embryo cDNA library. One isoform, RI, has previously been shown to be active at repressing cysteine protease activity in insect digestive tracts (Hines *et al.*, 1991), and is, in common with a second isoform N2, induced by wounding, suggesting a protectant role. The third isoform, L1, has been shown (as a recombinant protein) to be active against the major thiol-endopeptidase in mung bean seedlings suggesting a role as a regulator of endogenous protease activity. To confirm this diversity of function, Zhao *et al.* (1996) showed that both wound-induced isoforms (N2 and R1) were substantially more effective at retarding gut cysteine proteinase activity of the western corn rootworn and Colorado potato beetle when compared with the constitutively-expressed L1 isoform. Kouzama and colleagues purified two distinct cystatins from seeds of sunflower and proposed that one, Sca, may function as a regulator of endogenous cysteine proteinase activity while Scb might be involved in the protection of the seed against exogenous proteinase activity (Kouzuma *et al.*, 1996).

Although not characterised at the subcellular level, cystatins are known to be intercellular in localisation. However, one recent report describes a cysteine proteinase inhibitor which exists as an insoluble protein in the cell walls of carrot seeds located both in the embryo and the inner edge of the endosperm (Ojima *et al.*, 1997). The insolubility of the protein has made accurate determination of the inhibitory characteristics difficult, but because of the location of the inhibitor in the seed, it most likely functions as a regulator of endogenous proteinases.

Proteinase Inhibitors in Apple Seeds

A range of proteinase inhibitors have been characterised in mature apple seed using sensitive detection methods based on fluorogenic protease substrates (Table 2). The papain inhibitors identified ranged in abundance from 0.34 µg/g seed (Pap2-1) to 30.8 µg/g seed (HMW Pap) which compares with reported ranges of serine proteinase inhibitors of 0.25 mg/g for mungbean seed, 1.5 mg/g for chickpea seed and 3.6 mg/g for kidney bean

Table 2. Summary of proteinase inhibitors identified in mature seeds of apple (from Ryan *et al.*, 2000).

Inhibitor Designation	Proteinase Inhibited	Molecular Weight (kDa)	Concentration (µg/g seed)
HMW Pap	Papain	40.0	30.8
LMW Pap1	Papain	10.7	1.07
LMW Pap2	Papain	nd[1]	nd
LMW Pap2-1 (derived from Pap2-1)	Papain	11.5	0.34
LMW Pap2-2	Papain	11.6	6.7
Trp	Trypsin	7.7	2.3

[1]Not determined

seeds (Richardson, 1977). More specifically, the Kunitz and Bowman-Birk inhibitors have been calculated to comprise 3 - 4 mg/g of soybean seed (Tan-Wilson *et al.*, 1982). For the cysteine proteinase inhibitors, the well characterised cystatin purified from corn endosperm (CI-4a) (Abe *et al.*, 1980; Abe and Whitaker, 1988; Abe and Arie, 1991) which is an inhibitor of the major thiol protease responsible for the degradation of the major storage protein, zein, has been calculated to occur at a concentration of 6 μg/g endosperm tissue (Abe and Whitaker, 1988). A cystatin purifed from red kidney seeds, which also regulates endogenous cysteine proteinases, has been shown to occur at a concentration of 1 μg/g seed (Brzin *et al.*, 1998). The cystatins identified in apple seeds, therefore, are present at levels which are akin to inhibitors of endogenous cysteine proteinases in seeds of other species, and are substantially diminished when compared with values reported for serine proteinase inhibitors in legume seeds.

A serine (trypsin) proteinase inhibitor has also been identified in mature apple seeds (Table 2). This inhibitor occurs at a concentration of 2.3 μg/g seed which is also substantially less than the major serine proteinase inhibitors in legume seeds, for example, suggesting that this protein inhibits endogenous serine proteinases. Perhaps of more significance is that its discovery invites a search for the matching proteinase and asks: what function do such proteinases have in the mature seed of apple? The elucidation of such interactions will add to our understanding of the function of proteinase inhibitors in regulating endogenous proteinases in seeds.

Summary

Evidence for each of three postulated functions for proteinase inhibitors in seeds has been reviewed. We contend that the evidence which supports these proteins as seed protectants is convincing. These proteins are potent inhibitors of proteinase activity of insect and microbial origin, and their abundance in terms of representing a major portion of stored protein in the seed, is in excess of that required to inhibit endogenous proteinases. However, a detailed study on the occurrence of specific inhibitors and the matching spectrum of proteinases from either pests of these seeds, or from insects or microbes containing susceptible enzymes has yet to be undertaken. This is particularly so for seeds of species which occur in natural ecosystems.

The abundance of these inhibitors has also been the basis for proposing that they act as storage proteins in seeds. As well, earlier experiments suggested that the timing of maximum proteinase inhibitor content coincided with maximum proteolysis during germination. It is likely that the very abundant inhibitors in seeds do play a dual role. In the dry seed, they can act as protectants against proteinases of external origin (insects, microbes). However, once reserve mobilisation is initiated, then these proteins do represent a significant source of stored nitrogen.

It is apparent that to make definitive progress on the elucidation of function of proteinase inhibitors it is important to work with purified proteins. More recent studies in which purified proteins are used, in concert with more sensitive assays to detect proteinases (and thus their inhibitors) have revealed a myriad of proteinase inhibitors of varying abundance and (probably) a matching set of proteinases in seeds. These studies also indicate that the less abundant inhibitors function as regulators of the endogenous proteinases. More commonly, these are identified as cysteine proteinase inhibitors (phytocystatins) which may reflect the importance of cysteine proteinases in plants. In the authors' work on apple seeds, the less abundant inhibitors have, in the main, been shown to the phytocystatins (Ryan *et al.*, 2000).

The advent of molecular biology and functional genomics will have a significant impact on our understanding on the role of proteinase inhibitors in seeds. Sequencing of the *Arabidopsis* genome has already identified a host of inhibitors and proteinases which may occur in such low abundance, or are in association with other proteins, that they escape discovery using standard biochemical techniques. However, once identified, there is the prospect of interesting biochemistry, in association with gene expression studies, to determine the role of these proteins. Such approaches will, in turn, have a considerable impact on our knowledge on how the processes of seed development and germination are regulated.

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