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A CYSTEINE PROTEINASE INHIBITOR PURIFIED FROM APPLE FRUIT

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Key Word Index—*Malus domestica*; Rosacae; apple-Royal Gala; proteins; cyteine proteinase inhibitor; papain/ficin/bromelain inhibitor.

Abstract—A cysteine proteinase inhibitor has been purified from immature fruit of *Malus domestica* (var. Royal Gala). The M_r of this apple cystatin is estimated to be 10 700 by MALDI–TOF mass spectrometry, 11 300 by SDS–PAGE and 11 000 by gel filtration. It is a relatively strong inhibitor of papain with a K_i value of 0.21 nM and also inhibits ficin and bromelain but not cathepsin B. An amino acid sequence was obtained from a peptide produced by trypsin digestion of the inhibitor. Comparison with other plant sequences shows a high degree of homology with other phytocystatins. As the single cysteine proteinase inhibitor detectable in immature apple fruit (5–8 mm diameter), levels of 83.3 pmol/g FW were determined. In larger fruit (up to 16 mm diameter) significantly less inhibitor was present (6.9 pmol/g FW). Given these low levels, it is postulated that this inhibitor has an endogenous role in apple fruit development rather than one of protection against pest or microbial attack.

INTRODUCTION

Cysteine proteinase inhibitors in animals have been classified into three distinct families on the basis of M_r, the number of disulphide bonds, subcellular localisation and primary structure characteristics. Family 1 cystatins have a M_r around 11 000 and lack a disulphide bond. Cystatins in family 2 contain two disulphide bonds and their M_rs are around 13 500. Family 3 are a kininogen family [1].

Among plant cystatins only the cystatins from rice (oryzacystatins) have been well characterised. From genomic sequences, the rice family are considered to resemble the family 2 cystatins of animal origin, although they have no disulphide bonds or cysteine residues. However, the genomic organisation of oryzacystatin, and another cystatin from corn, differs markedly from animal cystatins giving rise to the suggestion that these should form a new family designated *phytocystatins* [2, 3].

The complete amino acid sequence has been elucidated for a number of phytocystatins including oryzacystatin I [4] and II [5], as well as cystatins from cowpea seeds [6], avocado fruit [7] and corn kernel tissue [8]. These sequences all contain the highly conserved putative binding region "QVVAG" which characterises cystatins whether they have a plant or animal origin, and are structurally similar. The level of homology between cystatins from seeds of different species is no different from the homology observed between seeds and fruit cystatins from the same species suggesting a conserved function for the protein in plants [7].

We are interested in the roles of proteinase inhibitors in plants, particularly with respect to the protection of fruit crops from fungal, bacterial and insect attack. Tobacco plants transformed with genes coding serine proteinase inhibitors have been shown to confer insect pest resistance to the transformants [9–11]. Insect pests have also been shown to have cysteine proteinases in their gut [12], and oryzacystatin inhibits the growth of *Sitophilus zeamais* by blocking its digestive cysteine proteinases [13]. These results show that the identification and characterisation of other phytocystatins should reveal further candidates to confer insect pest resistance to crop plants.

We have purified and characterised a lower M_r cysteine proteinase inhibitor from the immature fruit of apple *Malus domestica* (var. Royal Gala).

RESULTS AND DISCUSSION

Purification of a cysteine proteinase inhibitor

The level of proteinase inhibition in whole immature apples (< 5 mm in diameter) was very low (Table

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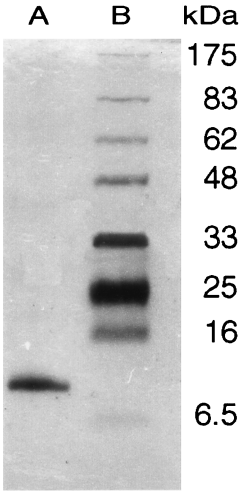

Fig. 1. Silver-stained SDS–PAGE separation of 0.1 μg of HPLC purified apple cystatin (A) and M_r standards (B).

1 and 2). No trypsin inhibition could be measured (limits of detection <1.0 pmol/g FW) while only a low level (>10 pmol/g FW) of cysteine proteinase inhibition was detectable. Purification of a cysteine proteinase inhibitor was achieved by acidifying the extract, pH adjustment by Sephadex G-25 gel filtration chromatography followed by papain affinity chromatography and reverse phase HPLC. Inhibitory activity was only detectable after elution of an inhibitor from the papain affinity column. A single peak of inhibitory activity was determined after both affinity column chromatography and reverse-phase HPLC (data not shown).

Purity of the inhibitor was determined using SDS–PAGE which revealed a single protein with an estimated M_r of 11.4×10^3 (Figure 1). As only one cysteine proteinase inhibitor is present in apple fruit <10 mm in diameter, G75 Superdex (G–75) gel filtration column chromatography was used with extracts after pH adjustment with Sephadex G-25 to determine a native M_r. A single peak of papain inhibitory activity was detected with a M_r of *ca* 11.1×10^3 (Figure 2). A definitive M_r estimation was determined by MALDI-TOF mass spectrometry and found to be 10.7×10^3 (Figure 3).

The dissociation constant of the inhibitor binding to the proteinase papain was determined by titration of a known amount of papain against a serial dilution of the inhibitor solution (Figure 4A). The K_i of the inhibitor was $0.42 \pm 0.12 \times 10^{-9}$ M, indicating a relatively strong inhibitor of papain [14]. The dissociation constant for the potato tuber cysteine proteinase inhibitor was substantially smaller at 7×10^{-11} M [15] while that from rice seeds (oryzacystatin I) was larger at (3×10^{-8} M) [4].

Inhibition of a range of other papain-like cysteine proteinases were also tested with the apple cysteine proteinase inhibitor. Equimolar quantities of bromelain and ficin equivalent to that of papain, were all significantly inhibited (Figure 4B), while proteolytic activity of cathepsin B was unaffected.

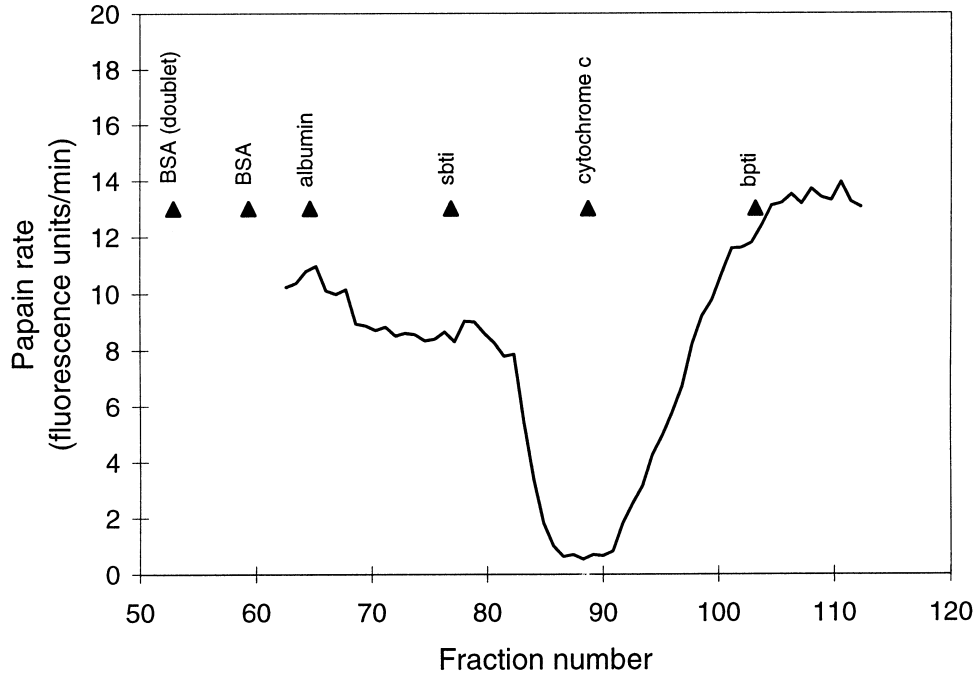

Fig. 2. Chromatography of the concentrated apple protein extract using Sephadex G-75. Fractions were assayed for papain activity as described in the text. The elution of proteins of known M_r are indicated. BSA = bovine serum albumin; BPTI = bovine pancreatic trypsin inhibitor; SBTI = soybean trypsin inhibitor.

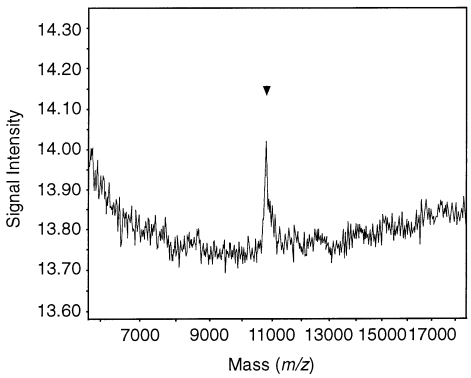

Fig. 3. MALDI-TOF Mass Spectrometry spectra indicating M_r of the purified apple cysteine proteinase inhibitor (arrowed).

Amino acid sequencing of the purified inhibitor

Initial attempts at obtaining N-terminal sequence from the purified inhibitor were unsuccessful, but one peptide generated by trypsin digestion yielded reliable sequence data. The sequence obtained from apple fruit was compared with a number of other plant sequence and the overlapping regions aligned (Figure 5). The greatest homology with the apple cystatin sequence is with the deduced amino acid sequence from a cDNA obtained from unripe pear mesocarp (Genbank accession number U82220) with 93% identity over the region of overlap Figure 5. Sequences from soybean [16], avocado [7], corn [8], rice [4, 5] and cowpea [6] all share *ca* 50–60% of identical residues with the apple sequence. Full length sequences have been reported for a number of plant cysteine proteinase inhibitors. The apple peptide matches these other sequences close to their amino termini and upstream away from the highly homologous QVVAG putative binding region for cystatins which typically lies close to the midpoint between the N-terminus and C-terminus. There appears to be little significant change in the level of identity when comparing cystatins of seed origin with those from fruit [13]. In addition to the apple sequence, both the avocado and pear sequences share a similar identity to the seed cystatins listed.

The amino acid analysis revealed differences in the composition of the apple cystatin when compared with the pear, avocado and soybean cystatins which were the most identical sequences to the apple, but as a group were remarkably similar in amino acid composition (Table 3). However, some differences were apparent. The apple cystatin has a greater abundance of serine, glycine, and tyrosine as well as a slight reduction in the number of valine, lysine and tryptophan residues.

Occurrence of cysteine proteinase inhibition during apple fruit development

To quantify the amount of apple cysteine proteinase inhibitor in apples of different sizes (< 5 mm, 5–10 mm, 10–15 mm, > 50 mm), the inhibitor was purified using the procedure outlined previously up to and including the affinity chromatography step, the eluate concentrated and the amount of inhibitor quantified by titration against papain. There was a significant variation in the extractable amount of inhibitor from the different size classes of fruit as well as from the different tissue types from the older apples (Table 1). The inhibitor is present in low levels throughout the earliest stage (< 5 mm diameter) of apple fruit development. In contrast, the yield from apples between 5–10 mm was the highest on a per g fresh weight basis, and this also translated into the highest estimation of PI per apple. The reduction in inhibitor per g fresh weight observed as fruit matures is most likely due to their greater size and therefore is simply a dilution effect. Larger apples (> 50 mm diam.) were divided into three tissue types i.e. peel, cortex and seeds in order to estimate the relative distribution of the PI throughout the apple (Table 2). The greatest yield was from these immature seeds which had *ca* 56-fold as much inhibitor extracted as the cortex tissue. Although there was more inhibitor in the peel than the cortex, it was still 18-fold less in these tissues than that extracted from the seeds. However, since papain affinity column chromatography only was used (without subsequent HPLC purification), we cannot say unequivocally that the inhibition detected in mature fruit is comprised solely of the inhibitor purified from immature fruit. We do know that the seeds contain other inhibitors, and two other cysteine PIs as well as two trypsin inhibitors have been identified thus far (data not shown).

Apple fruit develop rapidly, first undergoing a period of cell division and then a period of rapid cell expansion during which there is little further cell division [17]. The peak of inhibitor abundance at early stages of apple growth (5–10 mm diameter) is low and although there is a 50-fold higher level in the seeds of

Table 1. Levels of the purified cysteine proteinase inhibitor in immature apples. The value of PI/apple takes into account the different sizes of the apples

Diameter	< 5 mm	5–10 mm	10–16 mm
pmol/g FW	18	84	7
pi/apple (relative)	2	40	12

Table 2. Levels of cysteine proteinase inhibition in different tissues of 50 mm diameter apples

	peel	cortex	seeds
pmol/apple	182	63	1667
pmol/g FW	18	6	329
relative concn	3	1	56

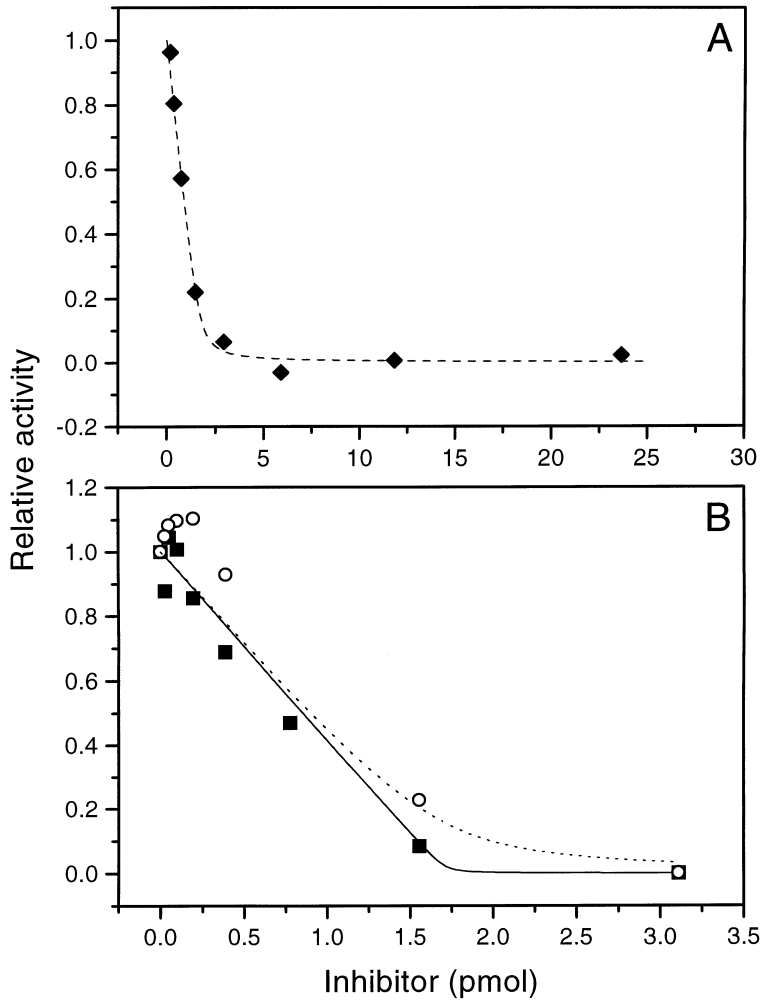

Fig. 4. Titration of purified apple cystatin against Papain (A), and against Ficin ○ and Bromelain ■ (B). The line fitted to the papain data was derived from the calculated dissociation constant, K_i.

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Apple (fruit)              DNQGSANSVETESLA
                                 *      **
Pear (fruit)      MAAVGAVRDNQGVANSVETESLARYAVDEHNKKEN...   35
Mustard           MAMLGGVRDVPSNENSVEVESLARFAVDEHNKKEN...   35
Soybean      AALEKVQELGGITDVHGAANSVEINNLARFAVEEQNKREN...   40
Avocado (fruit)    PLLGGVRDV-PDHNSAETEELARFAVQEHNKKAN...   33
Cowpea            MAALGGNRDVAGNQNSLEIDSLARFAVEEHNKKQN...   35
Rice (OcI)    MSSDGGPVLGGVEPVGNENDLHLVDLARFAVTEHNKKAN...   39
Corn               AALGGNRDVPANENDLQLQELARFAVNEHNQKAN...   34
                                 *      *** **
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(OcI = oryzacystatin I)

Fig. 5. Comparison of the amino acid sequence of the apple cysteine proteinase inhibitor with homologous predicted amino acid sequences of other plant cysteine PIs. (Positions with * indicate identical amino acid residues in all the sequences). The accession numbers for the sequences used for comparison are U82220, S65071, U51854, JH0269, S39506, A28464, A38375, respectively.

Table 3. Amino acid composition of various cysteine proteinase inhibitors. The apple content was measured as described in the text, while the amino acid contents for the other inhibitors was calculated from the reported amino acid sequence (avocado; [7]) or from the sequence deduced from the cDNA (pear; Genbank accession no. U82220; soybean; [16])

Amino acid	Cystatin Source Apple	Avocado	Pear	Soybean
D/N	6	8	11	11
E/Q	9	16	14	18
G	11	5	5	5
H	1	2	2	1
R	3	4	3	4
T	4	5	3	3
A	6	9	8	10
P	3	3	2	1
Y	12	3	3	3
V	5	12	15	14
M	1	1	2	0
I	3	2	1	5
L	4	7	5	9
F	2	4	3	3
K	4	10	11	8
W	—	0	2	2
C	—	0	0	0
Total	83	100	95	103

(— not measured)

the larger apples, the amount of inhibitor found in all the tissues examined is much lower than those found in plants where cystatins are thought to have a pest resistance role (up to 0.6 mg/gFW in the leaves of potato plants [1]). These low levels suggest a metabolic regulatory role for the inhibitor during the early stages of fruit development. However, if the accumulation of the PI in the seeds of developing apples continues with fruit maturation, then the concentration of the inhibitor in mature apple fruit may approach the levels of other cysteine PIs reported in potato plants, thus suggesting a role for this PI in resistance of the apple fruit to insect or microbial attack. If there is no further increase in PI conc in mature seeds then it is more likely to have an endogenous function in the physiology of apple fruit development, perhaps in the regulation of endogenous seed cysteine proteinases during seed maturation. However, until the nature of cysteine proteinase inhibition in these tissues is characterised more fully, the functions of these proteins in mature apple fruit can only be surmised.

EXPERIMENTAL

Plant material

Apple fruit of the species *Malus domestica* (var. Royal Gala) were collected from the HortResearch Kumeu Research Orchard, Auckland, New Zealand. Fruit were separated into size classes based on fruit diam., frozen in liquid N_2 and stored at -80° until used. Larger fruit (>50 mm in diameter) were dissected into peel, cortex and seeds before freezing.

Preparation of papain affinity column

5 ml Affigel 10 (Biorad Laboratories, Hercules, CA, USA) was washed with copious quantities of ice-cold H_2O and then equilibrated with 100 mM MOPS (pH 7). Papain soln was added to the gel slurry to a concn of *ca* 3.5 mg.ml^{-1}, and the mixture agitated gently for 2 hr at room temp. The reacted gel mix was transferred to a small column and washed copiously with wash buffer (10 mM MOPS (pH 7) containing 500 mM NaCl, 2 mM EDTA and 2 mM DTT), followed by 20 vol of elution buffer (50 mM HCl) and then 20 vol of wash buffer.

Purification of cysteine proteinase inhibitor

Whole immature fruit ($\leqslant$15 mm diam.) were ground to a powder under liquid N_2 with the addition of solid PVPP. The powder was added to cold extraction buffer (100 mM bis-tris propane (pH 7.0) containing 2 mM EDTA, 2 mM EGTA, 1% (v/v) Tween 20, 10 mM DTT, 1% (v/v) 2ME, 0.1% (w/v) PEG6000, 1 mM PMSF) to provide a final concn of 10 mg/ml hydrated PVPP. The resulting slurry was ground in an ice-cold mortar and pestle before filtering through Miracloth™ (Calbiochem–Novabiochem Corp, LaJolla, CA, USA). The filtrate was centrifuged at 10 000 *g* for 20 min at 4°, the supernatant adjusted to pH 3 with 36% HCl. Following further centrifugation at 10 000 *g* for 30 min at 4°, the extract was adjusted to pH 8 by application to a 5×20 cm Sephadex G-25 column (medium grade; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) previously equilibrated with 100 mM Tris-HCl, pH 8. Fractions containing protein (Bradford micromethod; Biorad Laboratories) were pooled for application to a papain affinity column. Protein which did not bind to the affinity column was washed through the column. Elution buffer (50 mM HCl) was added to the column until the eluent had reached pH 2 at which point the flow was stopped for 10 min. Fractions (1 ml) were then collected and immediately neutralised with 3 M Tris HCl (pH 10). Fractions in which papain inhibition was detected were concd and applied to a Vydac C-18 reverse-phase column (Alltech, Deerfield, IL). Proteins were eluted with a gradient from 20% (v/v) MeCN, 0.1% (v/v) TFA to 80% (v/v) MeCN, 0.1% (v/v) TFA, and fractions containing proteinase inhibitory activity were pooled.

Proteinase inhibition assays

Commercially available proteinases (papain, chymopapain, bromelain, cathepsin B, and ficin) were used in the assays which were performed in a 96-

well white fluorescence micro-titre plates (A/S Nunc, Roskilde, Denmark). Appropriate proteinase concs were determined by titration. To determine K_i values a single concn of proteinase (eg 0.04 μg papain) was incubated with a range of inhibitor concs for 10 min at 25°. This mix was buffered to a final concn of 100 mM MOPS (pH 6.8) and the reaction initiated by the addition of benzyloxycarbonyl-Arg-7-amido-4-coumarin (Z-R-AMC, BACHEM Feinchemikalien AG, Bubendorf, Switzerland) to give a final concn of 2 mM. The reaction was monitored using a Fluoroscan II (Labsystems, Helsinki, Finland) using an excitation wavelength of 355 nm and emission wavelength of 460 nm. Rates were calculated from linear regression of the data using in-house software described in ref. [18].

SDS-PAGE

SDS–PAGE was performed using in the Bio-Rad Mini-PROTEAN II system with a 4% stacking gel and a 10% separating gel [19]. Bio-Rad broad range stained marker proteins were used to indicate M_r.

Superdex R G75 chromatography

Pooled fractions from the G25 column containing protein (Bradford micro-method; Biorad Laboratories) were reduced to about 1 ml using a Microcon concentrator and a YM3 membrane (Amicon Division, Beverly MA, USA). The crude protein soln was applied to a G75 Superdex (prep grade, Pharmacia-LKB) previously equilibrated with 100 mM Tris-HCl, pH 8.0, and fractions assayed for inhibition of papain.

M_r Determinations

A Finnigan Lasermat 2000 matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass analyser (Finnigan MAT, Hemel Hemstead, England) was used to determine the M_r of the inhibitor [20].

Amino acid analysis

For amino acid analysis, samples were submitted to the Protein Microchemistry Facility, Department of Biochemistry, University of Otago. Analyses were undertaken as described [21].

Protein sequencing

Analysis of N-terminal sequence of the apple cysteine proteinase inhibitor was unsuccessful suggesting that the N-terminal amino acid was blocked. Therefore a sample was digested overnight with 1 μg trypsin in 10% (v/v) MeCN, 100 mM Tris, pH 8.0, 0.02% (v/v) Triton X100, the digestion separated by HPLC on a Brownlee RP-300, 220 × 2.1 mm, 7 μm column pre-equilibrated at 10%B (A = 0.1% TFA, B = 0.0825% TFA/80% MeCN). The peptides were eluted with a 10–70% gradient over 45 min and the 3rd major peak sequenced on an Applied Biosystems Procise sequencer (Applied Biosystems, Foster City, CA, USA). The two other major peaks did not produce reliable data and it was assumed that they both contained the N-terminal which was blocked.

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