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Identification and Characterisation of Proteinase Inhibitors and Their Genes from Seeds of Apple (*Malus domestica*)

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Trypsin and papain proteinase inhibitors have been identified and purified from aqueous extracts of apple seeds (*Malus domestica*). Superdex G75 gel filtration chromatography identified a higher molecular weight (HMW) papain inhibitory fraction (22–26 kDa) and a lower molecular weight papain and trypsin inhibitory fraction (6–12 kDa). The lower molecular weight fraction was separated into a trypsin inhibitor (designated Trp1) and early (designated Pap1) and late (designated Pap2) eluting papain inhibitors after anion exchange (Hitrap SP) chromatography. For Pap2, two inhibitory peaks (designated Pap2-1 and Pap2-2) were identified after further anion exchange (Resource S) chromatography. Each of these lower molecular weight inhibitors was purified by reverse phase HPLC to homogeneity as determined by SDS-PAGE and by mass spectrometry. The HMW papain inhibitory fraction was purified further by anion-exchange (Hitrap Q followed by Resource Q) column chromatography where a minor inhibitor (HMWPap1) and major inhibitor (HMWPap2) fraction were identified. The relative abundance in seeds of apple and the spectrum of proteinase inhibition has been determined for all of these inhibitors. Reverse-phase HPLC separated HMWPap2 into a minor (HMWPap2-1) and a major (HMWPap2-2) inhibitory fraction, and SDS-PAGE and mass spectrometry confirmed that HMWPap2-2 was purified to homogeneity. Amino acid composition data were obtained from Trp1, Pap1, Pap2-2, and HMWPap2-2, and N-terminal sequence data from Trp1, Pap2-1, Pap2-2, and HMWPap2-2, with two of these sequences (Pap2-2 and HMWPap2-2) perfectly matching predicted protein sequences based on EST sequences from an apple database. The relationship of these inhibitors with those of other species is discussed.

Key words: cystatin, *Malus domesticus*, proteinase inhibitor, Rosacea, seeds, trypsin.

Abbreviations: K_I, equilibrium inhibition constant; PI, proteinase inhibitor; Z, benzyloxycarbonyl.

Seeds of higher plants are a rich source of proteinaceous inhibitors of proteinases, with the serine proteinase inhibitors the most extensively characterised thus far (*1–4*). Cysteine proteinase inhibitors (cystatins) represent the next best characterised group in seeds including those from a number of plant species such as rice (*5*) corn (*6*), cowpea (*7*), sunflower (*8*), sorghum (*9*), soybean (*10*), carrot (*11*), bean (*12*, *13*) and apple (*14*). In comparison, fewer inhibitors of the aspartic (*15*, *16*) and metallo (*17*, *18*) proteinases have been identified in seeds.

Cystatins of seed origin have been well characterised in rice (oryzacystatins), and have been shown to resemble the family 2 cystatins of animal origin, although the oryzacystatins have no disulphide bonds or cysteine residues. Further, the amino acid sequence for cystatins from seeds of rice (*19*, *20*), cowpea (*7*) and corn kernel tissue (*21*) contain the highly conserved putative binding region QXVXG that is also characteristic of animal cystatins. Cystatins of plant origin are also identified by the plant-specific consensus sequence (LVI)-(AGT)-(RKE)-(FY)-(AS)-(VI)-X-(EDQV)-(HYFQ)-N, found within the amino-terminal alpha-helix (*22*). These proteins often have a diagnostic glycine near the N terminal (*23*) and a less conserved E-A-K-(VF)-W-V-K-P-W consensus at the second contact point with target proteinases. However, oryzacystatin, and another cystatin from corn, differ markedly from animal cystatins in their genomic organisation, suggesting that they may represent a new family, the phytocystatins (*24*, *25*).

In terms of function, proteinase inhibitors in seed tissues, particularly the serine inhibitors, have been proposed to act as protectants against insects or predation, especially since these proteins have been deployed widely as insect pest resistance factors (*26–31*). While genes coding for the cystatins have also been used successfully against insect pests in transgenic plants (*32*, *33*), other studies have shown that proteinase inhibitors may also regulate endogenous proteinases in seeds (*8*, *12*, *13*, *34–36*). Thus to increase our understanding of the role of cystatins in seeds, we set out to characterise a series of cystatins in seeds of apple.

We have analysed previously the proteinase inhibitors in pericarp tissue of apple fruit, and discovered only a single cystatin that occurs at a very low level (*14*). We

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suggested that because of the low level of the inhibitor, it may play a role in regulating an endogenous proteinase. In this study, we have now identified and characterised in detail a number of papain and trypsin inhibitors in seeds of apple, determined the relative abundance and spectrum of proteinase inhibition of each protein and identified genes for some of the inhibitors.

EXPERIMENTAL PROCEDURES

Plant Material—Mature apple fruit of the species *Malus domestica* (var. Royal Gala) were purchased commercially, and seed from the fruit removed and either used immediately or stored at –80°C until use.

Purification of Proteinase Inhibitors from Seeds—Typically, 70 g of whole seed (including the testa) were ground to a powder using a cryo-mill under liquid N_2. The powder was added to 200 mL of extraction buffer [0.1 M Bis-Tris Propane (pH 6.8), containing 2 mM EDTA, 2 mM EGTA, 0.2% (v/v) Tween 20, 10 mM DTT, 1% (v/v) 2-mercaptoethanol, 0.1% (w/v) PEG6000, 120 mg Pefbloc (Boehringer Mannheim, Mannhein, Germany), and 1 mg E64 (Sigma Chemical Company, St. Louis, MO, USA)]. Ten grams of solid polyvinylpolypyrrolidone (PVPP) was then added and the resulting slurry ground in an ice-cold mortar and pestle, extracted overnight with stirring at 4°C, before filtering through Miracloth™ (Calbiochem-Novabiochem Corp, La Jolla, CA, USA)]. The residue was re-extracted with 50 ml extraction buffer, the slurry filtered through Miracloth and the second filtrate combined with the initial filtrate. The pooled filtrates were then centrifuged at 20,000 ×*g* for 20 min and the supernatant adjusted to pH 3.0 with 1 M HCl. Following further centrifugation at 20,000 ×*g* for 20 min at 4°C, the extract was adjusted to pH 8.0 by chromatography through a 5 cm × 20 cm Sephadex G-25 column (medium grade; Amersham Pharmacia Biotech, Uppsala, Sweden), pre-equilibrated with 0.1 M Tris-HCl (pH 8.0), and protein was eluted at about 2 ml·min^{-1}.

Fractions with significant proteinase inhibitor activity were concentrated using an Amicon stirred cell concentrator with a YM3 membrane (Amicon Division, Beverly MA, USA), and the concentrate was then size-fractionated through a 1.8 cm × 40 cm Superdex G75 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 0.1 M Tris-HCl (pH 8.0) at 1 ml·min^{-1}. The G75 column was calibrated using a range of proteins of known molecular mass including BSA, myoglobin, cytochrome c and aprotinin.

Anion exchange chromatography was performed using two 5-ml capacity HiTrap Q columns (Amersham Pharmacia Biotech) arranged in series and each equilibrated with 0.02 M Tris-HCl (pH 8.0) at 4°C. Samples from the G75 column were diluted with water to 0.02 M Tris-HCl before being applied to the column at a flow rate of 1 ml min^{-1}. After exhaustive washing of the column, the retained proteinase inhibitors (PIs) were eluted within a gradient of 0–0.4 M NaCl in 0.02 M Tris-HCl (pH 8.0), over 190 ml. Active fractions were again diluted with water to a ionic strength equivalent to 0.02 M Tris-HCl and these inhibitors were purified further by chromatography through a 1-ml capacity Resource Q column (Amersham Pharmacia Biotech) and eluted, at 0.5 ml·min^{-1}, within a gradient of 0–0.4 M NaCl in 0.02 M Tris-HCl (pH 8.0), over 90 ml.

Cation exchange chromatography was performed using two 5-ml capacity HiTrap SP columns in series, each equilibrated with 0.02 M sodium acetate buffer (pH 5.0), at a flow rate of 1 ml·min^{-1}. Samples from G75 chromatography were diluted with 4 parts of water, and the pH was adjusted to 5.0 with 1 M acetic acid before application. Proteinase inhibitors were eluted within a gradient of 0–0.5 M NaCl in 0.02 M sodium acetate (pH 5.0), over 190 ml. Active fractions from this column were further purified by chromatography on a 1-ml capacity Resource S column equilibrated with 0.02 M sodium acetate (pH 5.0). Proteinase inhibitors were eluted, at 0.5 ml·min^{-1}, within a gradient of 0–0.175 M NaCl in 0.02 M sodium acetate (pH 5.0), over 90 ml.

Reverse-phase chromatography was performed using a 0.4 cm × 26 cm Jupiter C18 column (Phenomenex Inc. Torrance, CA, USA), equilibrated with 30% (v/v) acetonitrile and 0.1% (v/v) TFA in water. Proteinase inhibitors were eluted at 1 ml·min^{-1} within a 70 ml gradient comprising 30% (v/v) acetonitrile with 0.1% (v/v) TFA increasing to 60% (v/v) acetonitrile with 0.1% (v/v) TFA. Preliminary separations had established that all proteinase inhibitors detected eluted within 30% (v/v) to 60% (v/v) acetonitrile.

Proteinase Inhibition Assays—Papain [EC 3.4.22.2], chymopapain [EC 3.4.22.6], bromelain [EC 3.4.22.4], cathepsin B [EC 3.4.22.1], ficin [EC 3.4.22.3], chymotrypsin [EC 3.4.21.1], trypsin [EC 3.4.21.4] and pepsin [EC 3.4.23.1] were purchased from the Sigma Chemical Company and assays performed in 96-well white fluorescence micro-titre plates (A/S Nunc, Roskilde, Denmark). Papain, chymopapain, bromelain and ficin were assayed in 0.1 M MOPS (pH 6.8), while Cathepsin B was assayed in 0.1 M MES (pH 6.2). All enzyme reaction mixes contained 2 mM EDTA and 2 mM DTT. Substrates used were benzyloxycarbonyl-Arg-7-amido-4-coumarin (Z-R-AMC), H-Ala-Leu-Lys-AMC, Z-Gly-Gly-Arg-AMC, Z-Pro-Phe-Arg-AMC, Z-Phe-Arg-AMC, (Bachem Feinchemikalien AG, Bubendorf, Switzerland). Chymotrypsin and trypsin were assayed in 0.1 M Tris-HCl (pH 8.0), containing 2 mM CaCl2 using Z-R-AMC, Z-Gly-Gly-Arg-AMC and Z-Ala-Ala-Pro-Phe-AMC as substrates. Pepsin was assayed in 0.75% (w/v) lactate adjusted to pH 2.0 with HCl using the substrate Bodipy-casein (EnzChek™ protease assay kit green fluorescence, Molecular Probes, Eugene, OR, USA). Appropriate proteinase concentrations were determined by titration. To assay for the presence of proteinase inhibitors, a single concentration of proteinase (*e.g.*, 1–10 × 10^{-8} M papain) was incubated with 10 to 20 μl of inhibitor for 10 min at 25°C in 100 μl of assay mix. The reaction was initiated by the addition of substrate to give a final concentration of ~0.2 mM. The reaction was monitored using a Victor plate reader (EG&G Wallac, Finland) using an excitation wavelength of 355 nm and emission wavelength of 460 nm (10 nm band pass width filters). Rates were calculated from linear regression of the data using in-house software previously described (*38*). Binding affinities were established by performing the above assay at a range of inhibitor concentrations and at much lower enzyme concentrations

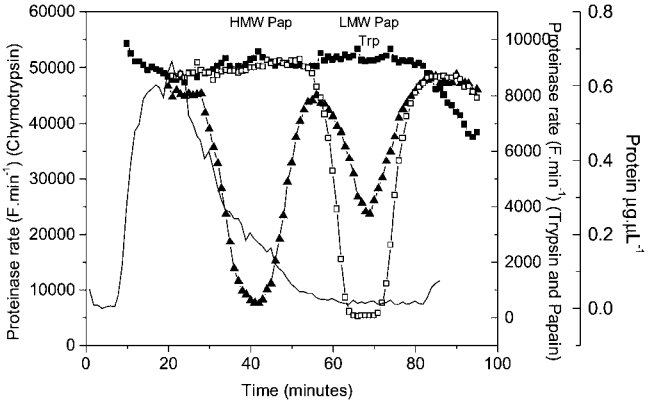

Fig. 1. **Superdex G75 separation of inhibitors from aqueous extracts of apple seeds.** (–) Protein elution determined as A_{280nm} ; (solid sqares) chymotrypsin activity; (solid triangles) papain activity; (open triangles) trypsin activity.

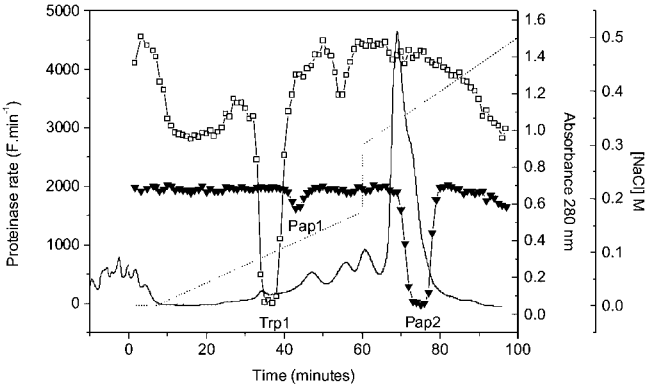

Fig. 2. **Separation, using Hitrap SP cation exchange chromatography, of the LMW papain- and trypsin-inhibitory fractions identified by Superdex G-75 gel filtration column chromatography.** (–) protein elution determined as A_{280nm} ; (solid triangles) papain activity; (open sqares) trypsin activity as fluorescence units (arbitrary change in fluorescence) per minute; (…) NaCl concentration.

(low nM concentrations) or by kinetic assays using a Perkin Elmer LS50B (*39–41*).

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE was performed using the Bio-Rad Mini-PROTEAN II system with a 4% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide separating gel using the Tricine-based buffer essentially as described by Schägger and von Jagow (*42*). A broad molecular mass range of stained marker proteins (Bio-Rad Laboratories, Hercules, CA, USA) were used to determine molecular mass. Gels were stained with either Coomassie blue or silver using standard techniques.

Mass Spectrometry—The molecular mass of purified inhibitors was determined either using a Finnigan Lasermat 2000 matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometer (Finnigan MAT, Hemel Hemstead, England) using the method of Hubbard and McHugh (*43*), or by *de novo* analysis of the mass spectra resulting from analysis on an LCQ Deca ion trap mass spectrometer fitted with an ESI interface (ThermoQuest, Finnigan, San Jose, CA, USA).

Protein Sequencing—N-terminal sequences of the proteinase inhibitors were determined using an Applied Biosystems Procise sequencer (Applied Biosystems, Foster City, CA, USA). Some samples were applied to a prosorb cartridge before sequencing, and some were treated with mild acid prior to sequencing. Homology searching was done using both the FastA and the Blast procedures (*44*).

Amino Acid Analysis—For amino acid analysis, samples were submitted to the Protein Microchemistry Facility, Department of Biochemistry, University of Otago, and the analysis was performed as described in (*45*).

RESULTS

Extraction and Purification of Apple Seed Proteinase Inhibitors—Seeds were extracted initially with an aqueous buffer, the supernatant reduced to pH 3.0 and precipitated proteins collected by centrifugation and the supernatant re-adjusted to pH 8.0 using Sephadex G-25 column chromatography. The concentrated G25 eluate was then applied to a calibrated Superdex G75 gel filtration column (Fig. 1). The elution pattern from the G75 column shows the bulk of the seed protein eluted first (as determined by absorbance at 280 nm), followed by two regions of proteinase inhibitory activity.

Papain inhibition separated into two fractions with molecular masses of 22–26 kDa [designated the higher molecular weight (HMW) fraction] and 6–12 kDa [designated the lower molecular (LMW) fraction], while trypsin inhibition eluted as one fraction with a molecular weight of 6–12 kDa. No chymotrypsin inhibitory activity was detectable when using less than 0.1 pmoles of chymotrypsin in the most sensitive assay, nor was any pepsin inhibitory activity detected at 11 pmoles of pepsin per assay.

To purify the LMW papain and trypsin inhibitory fractions further, the ionic strength of each Superdex fraction was reduced to about one third, the pH adjusted to 5.0 and the inhibitors chromatographed through a Hitrap SP cation exchange column (Fig. 2). The trypsin inhibitory activity (designated Trp1) eluted first (at 0.09 M NaCl), a minor papain inhibitory fraction (designated Pap1) eluted closely after (at 0.11 M NaCl), while the bulk of the papain inhibition (designated Pap2) eluted at a higher NaCl concentration (at 0.38 M NaCl). In other separations, when using larger amounts of seed material, a later eluting trypsin-inhibitory fraction can be detected at *ca.* 0.4 M NaCl, as well as two minor fractions which elute soon after Trp1 (data not shown). In these larger separations, extra papain-inhibitory fractions could also be detected, again eluting soon after Trp1 (data not shown). However, none of these additional inhibitory fractions were characterised further in this study.

The major trypsin (Trp1) and papain (Pap1, Pap2) inhibitory fractions identified in Fig. 2 were individually purified further using a Resource S cation exchange column (Fig. 3). For Trp1 (Fig. 3A) and Pap1 (Fig. 3B), only one activity peak eluted. However, for Pap2, two papain-inhibitory fractions (designated Pap2-1 and Pap2-2) eluted from the Resource S column (Fig. 3C).

The three papain-inhibitory fractions (Pap1, Pap2-1, Pap2-2) and Trp1 were purified further using reverse-phase HPLC and each eluted as single inhibitory fraction

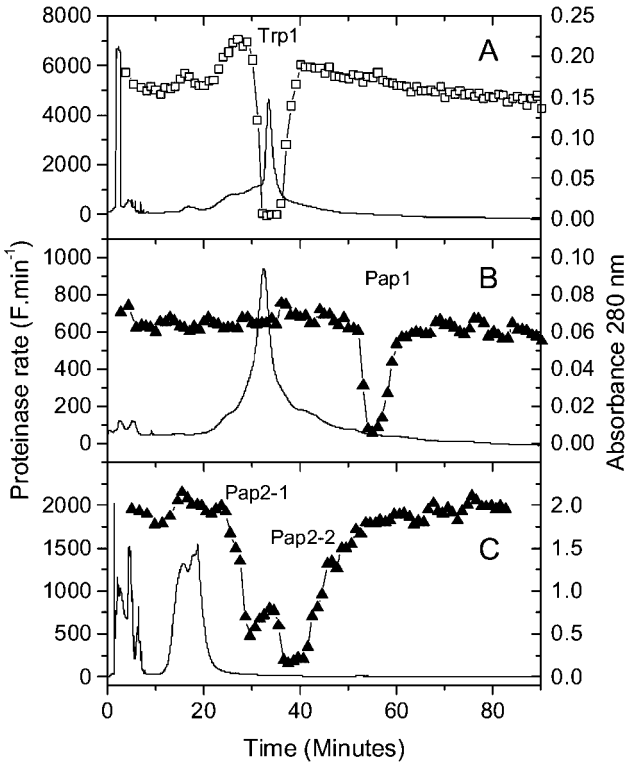

Fig. 3. **Separation, using a Resource S cation exchange chromatography, of the inhibitory fractions identified by the Hitrap SP column.** (A) Trp 1, (B) Pap 2–1 and (C) Pap 2–2. (–) protein elution determined as Absorbance at 280 nm; (solid triangles) papain activity; (open sqares) trypsin activity as fluorescence units (arbitrary change in fluorescence) per minute.

(data not shown). Electrophoresis of these inhibitory fractions (Trp1, Pap1, Pap2-1, Pap2-2) separated by reverse-phase HPLC revealed only one band after silver staining of the gel, indicating that each had been purified to homogeneity (data not shown).

The HMW papain inhibitor (HMWPap), identified after Superdex G-75 column chromatography (Fig. 1), did not bind to a SP-Hitrap cation exchange column at pH 5.0 and so was purified further by chromatography through a Hitrap Q anion exchange column (data not shown). One major inhibitory fraction was eluted (designated HMWPap2) as well as an earlier eluting minor peak (designated HMWPap1).

Determination of Abundance of Inhibitors in Apple Seeds—The amount of each inhibitor or inhibitory fraction was measured by titration of the eluate from the Resource S column (Trp1, Pap1, Pap2-1, and Pap2-2) or from the HiTrap Q column (HMWPap1 and HMWPap-2 inhibitory fractions). Each preparation was titrated against either papain or trypsin, and the amount of inhibitor was calculated from the linear extrapolation of the inhibition curve at low amounts of inhibitor. The results, presented as Table 1, show that the HMWPap2 inhibitory fraction (0.58 nmol/g of seed) and the Pap2-2 inhibitor (0.54 nmol/g of seed) comprise the major components of the papain inhibitors examined in apple seeds, although both occur with less abundance than the trypsin inhibitor, Trp1 (1.42 nmol/g of seed). The other inhibitors make up minor components of the inhibitory spectrum.

Table 1. **Abundance of different proteinase inhibitors in apple seeds.**

Inhibitor	Amount of inhibitor (nmol/g)	
	Papain	Trypsin
Trypsin	–	1.42
HMWP1	0.094	–
HMWP2[a]	0.58	–
Pap1	0.095	–
Pap2-1	0.027	–
Pap2-2	0.54	–

[a]Assayed as a composite of HMWPap2-1 and HMWPap2-2.

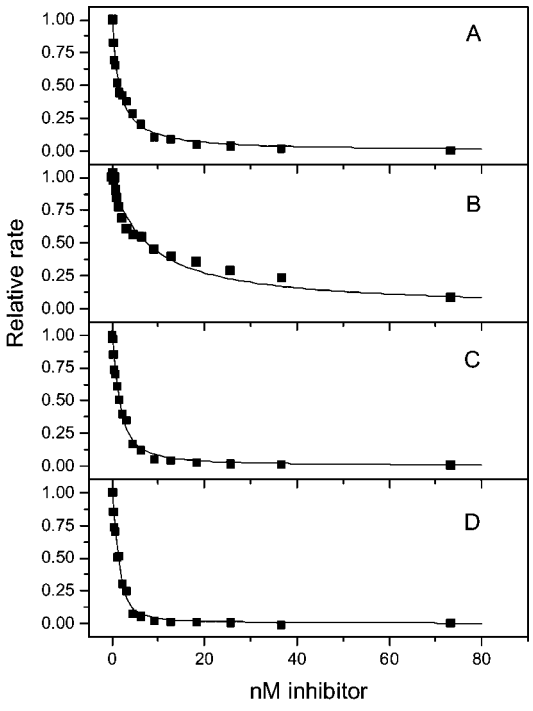

Fig. 4. **Inhibition of Chymopapain (A), Bromelain (B), Ficin (C) and Papain (D) by the Pap2 inhibitory fraction from seeds.** Proteinase concentration in the assay was 3 nM for all four proteinases.

Characterisation of Protease Inhibition—The papain and trypsin inhibitors were also titrated against a range of proteinases to determine their ability to inhibit different proteinases. Typical data for the inhibition of four peptidases (papain, bromelain, chymopapain and ficin) are shown for one inhibitory fraction, Pap2 (before it was resolved into Pap2-1 and 2-1) in Fig. 4, and a summary of calculated equilibrium inhibition constants (K_I) for the inhibitors tested against a range of enzymes is shown in Table 2. Generally, the cysteine proteinase inhibitors were most effective against papain (*i.e.*, low dissociation constant) and less effective against the other proteinases. The trypsin inhibitor, Trp1, showed a low nM inhibition constant against trypsin.

The HMW papain-inhibitory fraction, HMWPap2, was analysed further using a kinetic assay to determine the association and dissociation constants for the interaction of the inhibitor with papain assuming that this cystatin showed a simple one-step inhibition mechanism (*40*). A

Table 2. **Equilibrium binding constants for the inhibition of a range of proteinases by apple seed proteinase inhibitor preparations.**

Proteinase[b]	Inhibitor[a]			
	Pap1	Pap2	HMW Pap2	Trp1
	K_I (nM)[c]			
Papain	0.10 ± 0.03	0.34 ± 0.01	1.2 ± 0.3	–
Bromellain	48 ± 6	7.5 ± 0.6	3.8 ± 0.8	–
Chymopapain	0.73 ± 0.4	1.4 ± 0.2	3.3 ± 0.3	–
Ficin	0.26 ± 0.1	0.74 ± 0.1	1.1 ± 0.4	–
Tryspin	–	–	–	1.21 ± 0.37

[a]Inhibitors over a range of concentrations were incubated with the proteinases for 15 min at 25°C before relevant substrates were added and reaction rates measured. [b]Enzyme concentrations in the assays were: cysteine proteinases, 3 nM; trypsin, 0.42 nM. [c]Inhibition dissociation constants (K_I)were calculated using the non-linear fit package provided by Origin Graphics (Northampton, Ma, USA).

typical time course is shown as Fig. 5. The on rate constant (k_{ass}) was calculated as 1.1×10^6 $M^{-1} \cdot s^{-1}$ and the equilibrium inhibition (K_I) constant as 0.074×10^{-9} M. The calculated dissociation constant (k_{dis}) was 8.1×10^{-5} s^{-1}.

Mass Determination of the Purified Apple Seed Inhibitors—To ensure purity of the HMWPap2 inhibitory fraction prior to use for a mass determination, the preparation was re-chromatographed on a Resource Q anion exchange column, and again a broad papain-inhibitory fraction was recovered (data not shown). However, the HMWPap2 inhibitory fraction did separate into two discernable inhibitory fractions after separation using reverse-phase HPLC: a minor region (designated

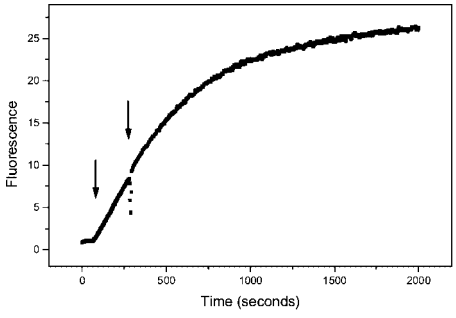

Fig. 5. **Time course of inhibition of papain by the HMWPap2 inhibitory fraction.** At the first arrow, papain (0.079 nM) was added, and at the second arrow, the inhibitor (2.3 nm) was added. Assays contained 0.9 mM of substrate (Z-Phe-Arg-AMC). In the absence of inhibitor, the assay continues as a linear extension of the straight line between the two arrows.

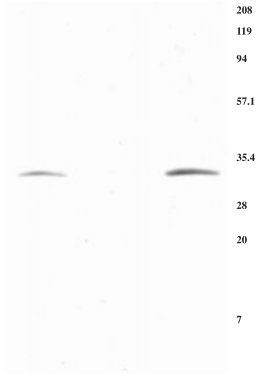

Fig. 6. **SDS PAGE of the HMWPap2 fractions.** HMWPap2-1 (right hand lane) and HMWPap2-2 (left hand lane) fractions purified by HPLC were separated by SDS PAGE as described in Methods. Numbers to the right are molecular weight times 10^{-3}.

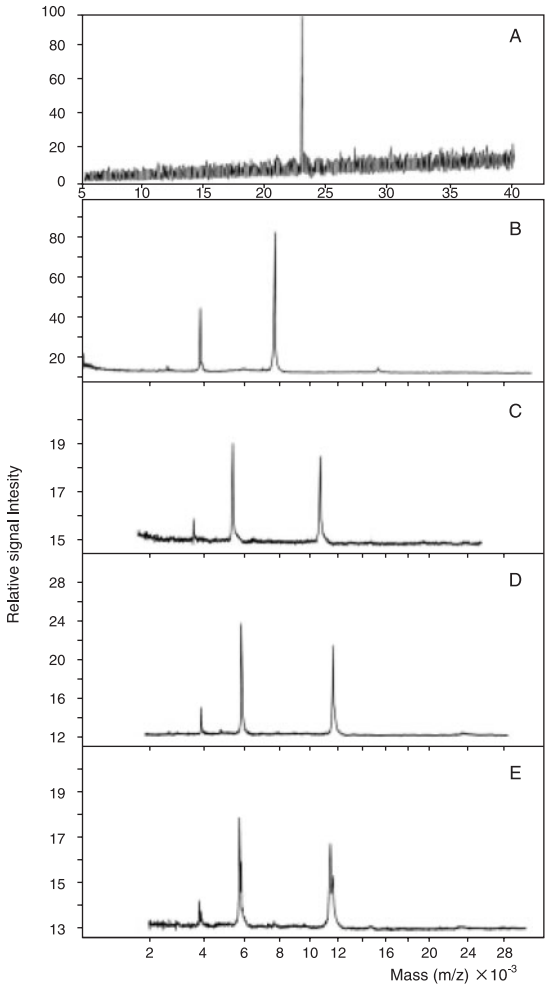

Fig. 7. **Mass spectral analysis of papain and trypsin inhibitors purified from apple.** (A) HMW papain inhibitor, HMWPap2-2 (B) Trypsin inhibitor, Trp1. (C) Pap 1. (D) Pap 2–1. (E) Pap2-2. A was determined by electrospray mass spectroscopy, while B to E were determined by MALDI-TOF. The three peaks in each of the MALDI-TOF graphs represent different charged variants of the same protein, with charges of 1+, 2+ and 3+. The machine-determined masses for these variants were within the machine specification of reproducibility (0.2%) when corrected for charge state.

Table 3. **Molecular mass and pI analysis of proteinase inhibitors from apple seeds.**

Inhibitor	Molecular Weight[a] ± Standard error	MW based on predicted peptide sequence from gene	pI based on predicted peptide sequence from gene
Trypsin Inhibitor	7,716 ± 0.2	Peptide sequence but no corresponding EST	Peptide sequence but no corresponding EST
Pap1	10,724 ± 1.4	No peptide sequence	No peptide sequence
Pap2-1	11,639 11,469	Version of Pap 2–2	Version of Pap 2–2
Pap2-2	11,633 ± 1.5	11,651.29	9.48
HMWPap2-2	22,591	22,584[b]	5.89[b]

[a]Values are means ± standard errors (n = 4) except for the HMWPap2-2 and Pap2-1 where only one measurement was done. All masses were determined using MALDI-TOF except for HMWpap2-2, which was determined using electrospray mass spectrometry (ES-MS) as described in "MATERIALS AND METHODS." [b]computed from residue 44 onwards (MATLGGVHE).

HMWPap2-1) eluting at 48 min, and a major inhibitory region (designated HMWPap2-2) that eluted at 51 to 52 min. SDS-PAGE and silver staining confirmed that each inhibitory region now comprised only a single band (Fig. 6). The HMWPap2-2 inhibitor was then subjected to electrospray mass spectrometry (ES-MS) and only one peak was discernable in the spectrum (Fig. 7A) The single peak confirmed the purity of the preparation and provided a molecular mass for the protein (Table 3).

MALDI-TOF mass spectrometry of the purified LMW fractions also showed clean peaks in each preparation (Fig. 7, B–D), except for Pap2-2 (Fig. 7E), which showed a small subsidiary peak, which may reflect a truncated inhibitor. The calculated molecular masses of these inhibitors are shown in Table 3.

Classification of the Purified Apple Seed Inhibitors Using N-Terminal Sequencing—A putative N-terminal sequence was determined for the LMW trypsin inhibitor, Trp1 (RPEVVGEHGEAVAAKIE). The sequence proved similar to a range of proteinase inhibitor I family trypsin and chymotrypsin inhibitors of seed origin (*46–48*) and an inhibitor from pumpkin phloem exudate (*49*). These sequences show 56 to 66% identity to the measured trp1 sequence over 15 to 17 amino acid residues. A predicted protein (AT5g43570) from the Arabidopsis database also showed a 50% identity over 16 amino acids at the N-terminal region of the protein.

No sequence was obtained from the Pap1 inhibitor, suggesting that the protein may be N-blocked, although treatment with acid did not release any sequence. A twenty-residue sequence was obtained from Pap2-2 (YGGMVGGRKEIENVKTNKEW). This sequence showed some identity to a translated mRNA sequence for an extracellular insoluble cystatin from Daucus carota (*11*) (identity = 55% over 18 residues), as well as to a cysteine proteinase inhibitor b (cystatin b) from sunflower (*8*) (identity = 53% over 15 residues). The sequence data obtained for Pap2-1 appeared to be a mixture of breakdown products of pap2-2. The predominant two sequences obtained were GGMV and YGGM, as well as minor amounts of GMVG and GRKE, and each of these sequences also occurs in Pap2-2.

The N-terminal of the purified HMWPap2-2 was also determined (PQVQDAANHAVKSLQQRSN) with the nearest genbank accession being a cysteine proteinase inhibitor from Glycine max (*10*). This showed 84% identity and 100% similarity over 19 amino acid residues.

Identification of Genes Corresponding to Pap2-2 and HMWPap2-2—The peptide sequences obtained from N-terminal sequencing were compared with the HortResearch EST database that contains over 100,000 EST sequences from libraries made to a range of tissues of apple. Comparison of the N-terminal sequence obtained from the trypsin inhibitor sequence, Trp1 revealed no matches in the EST database. For Pap2-2, a 100% match was observed with three sequences obtained from a vegetative bud library and one sequence from a seed library, and an alignment of the EST sequence obtained from seed library with the carrot cell wall cystatin is shown as Fig. 8A. Translation of the putatively corresponding EST sequence to Pap2-2 also allows the prediction of a molecular weight (11,651) and pI (9.48) (Table 3).

The HMW papain inhibitor, HMWPap2-2 displayed perfect matches with ESTs derived from a range of tissues including apple cortex (11 ESTs), floral buds (6 ESTs), as well as phloem, xylem, vascular buds, spur buds and senescing leaves (1 to 3 ESTs from libraries made to each tissue). No matches were found with EST libraries from seeds. An alignment of a selected EST predicted amino acid sequence with Soyacystatin is shown as Fig. 8B. It is noteworthy that the peptide sequence obtained from purified HMWPap2-2 aligns with the predicted peptide sequence from the EST over residues 167–185, thus representing an internal sequence. This suggests that the cystatin was sequenced at an internal cleavage site, and that the normal N-terminal residue was blocked (as no other underlying sequence was discernable). The residue immediately N-terminal to the observed sequence is aspartic acid, giving the potential cleavage site of Asp and Pro. It has been reported previously that the peptide linkage between Asp-Pro is particularly susceptible to mild acid hydrolysis (*50*), which is akin to the conditions (pH 3.0) that the protein was exposed to during purification. Translation of the putatively corresponding EST sequence to HMWPap2-2 also allows the prediction of a molecular weight (22,584) and pI (5.89) (Table 3).

Determination of Amino Acid Content—Amino acid composition data for the purified inhibitors Trp1, Pap1, and Pap 2-2 are shown in Table 4A and 4B [tryptophan (W) and cysteine (C) have not been determined]. The percentage composition of the Trp1 (Table 4A) is compared with the trypsin/subtilisin inhibitor sequence from Amaranthus seed (*47*) and the pumpkin phloem exudate

Fig. 8. **Alignment of the translated sequence for the putative apple seed cystatins from the HortResearch EST database with other cystatins.** (A) Alignment of the translated sequence of an EST (est 119789) that putatively corresponds to Pap2-2 with the carrot cell wall cystatin (gi|7438233|) The peptide sequence determined for the Pap2-2 inhibitor is shown in light grey on the EST sequence, and the three signature regions of cystatins discussed in the text are shown in dark grey on both sequences. (B) Alignment of the translated sequence of an EST (est187726) for an apple fruit cortex cystatin that putatively corresponds to HMWPap2-2 with Soyacystatin (gi|1944319|). The peptide sequence determined for the HMW papain inhibitor is shown in light grey on the EST sequence, and the three signature regions of cystatins discussed in the text are shown in dark grey on both sequences.

inhibitor (*49*). The amino acid composition of the two LMW papain inhibitors Pap1 and Pap 2-2 are shown in Table 4B, and their percentage composition is compared with an extracellular insoluble cystatin from Daucus carota (*11*), a cysteine proteinase inhibitor b (cystatin b) from sunflower (*8*), and the predicted amino acid composition of the EST that putatively corresponds to Pap2-2.

The amino acid composition of the HMW papain inhibitor, HMWPap2-2, was not determined. However, its composition can be calculated from the predicted amino acid composition of the EST that showed an identical peptide sequence to the HMW papain inhibitor. This composition is compared to the homologous Soyacystatin in Table 4C.

DISCUSSION

Seeds are a rich source of proteinase inhibitors in a wide variety of plants (*3*), and we have shown here the occurrence of at least five distinct inhibitors in seeds of apple. Four of these are papain inhibitors (designated Pap1, Pap2 and HMWPap1 and 2) and the other is a trypsin inhibitor, Trp1. Further, Pap2 and HMWPap2 may each comprise at least two variants (Pap2-1, Pap2-2; HMWPap2-1, HMWPap2-2). These proteinase inhibitors have been detected and characterised in mature apple seeds using sensitive detection methods based on fluorogenic proteinase substrates, which were essential because of the low levels of inhibitors in these seeds. The papain inhibitors identified ranged in abundance from 0.3 μg/g seed (Pap2-1) to 15 μg/g seed (HMWPap2), while the trypsin inhibitor characterised, Trp1, was present at 11 μg/g seed. To determine these amounts, the inhibitors were extracted and separated by chromatography, which may have resulted in losses. However, cystatins and plant family I trypsin proteinase inhibitors are extremely robust, and it is expected such losses would be small.

Table 4. **Amino acid composition of different proteinase inhibitors from apple.** (A). Amino acid content of the trypsin inhibitor, Trp1, in comparison to other similar inhibitors. Values for the number of residues are rounded to the nearest integer and are the means of four measurements. Standard errors were less than 0.6 in all cases except D/N (=1). (B) Amino acid content of Pap1 and Pap2-2 in comparison to other similar cystatins. Values for the number of residues are rounded to the nearest integer and are the means of four measurements (Pap2-2) or two measurements (Pap1). Standard errors were less than 0.5 in all cases except pap1 (<2). (C) Predicted amino acid content of an EST putatively corresponding to HMWpap2-2 in comparison to the Soyacystatin.

(A)

Inhibitor	Trypsin Inhibitor	Trypsin Inhibitor	Amaranthus seed	Squash phloem
Accession Number			P80211	S55591
Residues	Number	%	%	%
D/N	6	8.8	7.3	14.1
E/Q	12	16.8	11.6	15.6
S	2	3.3	4.4	7.8
G	9	12.7	7.3	7.8
H	2	2.1	1.5	0.0
R	7	9.1	11.6	7.8
T	3	4.7	5.8	1.6
A	6	9.1	5.8	4.7
P	3	4.8	5.8	7.8
Y	0	0.1	2.9	0.0
V	11	15.7	15.9	9.4
M	0	0.1	0.0	3.1
I	2	2.7	4.4	7.8
L	2	3.4	1.5	6.3
F	0	0.1	2.9	1.6
K	5	6.3	5.8	4.7
Total number of residues	70		69	64

(B)

Inhibitor	Pap1	Pap1	Pap2-2	Pap2-2	EST119789	Carrot cell walls	Sunflower seeds
Accession Number						BAA20464	Q10993
Residues	Number of Residues	%	Number of Residues	%	%	%	%
D/N	9	13.0	7	7.0	6.9	8.9	6.9
E/Q	15	21.5	19	17.8	16.7	14.3	10.9
S	7	10.1	6	6.0	5.9	7.1	5.0
G	13	18.9	13	12.3	11.8	12.5	8.9
H	2	3.5	3	2.4	2.0	3.6	1.0
R	6	7.9	7	6.9	6.9	0.9	3.0
T	4	6.1	3	2.6	2.0	2.7	7.9
A	7	9.6	4	3.8	2.9	10.7	6.9
P	4	5.5	3	3.3	2.9	2.7	3.0
Y	1	1.8	4	3.6	3.9	3.6	4.0
V	7	10.0	12	11.4	13.7	9.8	10.9
M	1	1.9	2	1.9	2.0	0.9	1.0
I	4	5.3	2	2.0	2.0	4.5	5.0
L	9	13.0	8	7.3	7.8	4.5	5.9
F	4	5.0	4	3.6	3.9	2.7	3.0
K	2	3.0	8	8.0	8.8	10.7	15.8
Total number of residues	95		105		103	112	100

(C)

Inhibitor	HMW papain inhibitor	Soyacystatin
Accession Number	EST 187726	BAA19608
Residues	%	%
D/N	7.4	10.6
E/Q	18.7	17.2
S	6.4	5.6
G	5.9	6.1
H	6.4	5.1
R	1.5	3.0
T	3.0	4.0
A	10.3	8.6
P	3.9	3.5
Y	1.0	1.0
V	10.3	9.1
M	2.5	1.0
I	1.0	2.5
L	7.4	8.6
F	3.4	4.5
K	9.4	9.6
W	1.5	1.5
C	0	0
Total number of residues	203	201

The abundance of the trypsin inhibitor is considerably lower than the reported ranges of seed serine proteinase inhibitors of 250 μg/g for mungbean seed, 1,500 μg/g for chickpea seed and 3,600 μg/g for kidney bean seeds (*1*). Also, the Kunitz and Bowman-Birk inhibitors have been calculated to comprise 3–4 mg/g of soybean seed (*51*). For the cysteine proteinase inhibitors, the well-characterised cystatin purified from corn endosperm (CI-4a) (*6*, *34*, *52*), 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 (*6*). A cystatin purified from seeds of red kidney bean, which also regulates endogenous cysteine proteinases, has been shown to occur at a concentration of 1 μg/g seed (*12*). 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. The low values for both the serine and cysteine proteinase inhibitors when compared with other seeds, suggests that they may not serve a defence function as postulated for many seed PIs.

The ability of these various inhibitors to bind to proteinases was measured using equilibrium binding assays. The measured K_I for Trp1 against trypsin (1.2 nM) compares favourably with that reported for the proteinase inhibitor 1 from *Amaranthus* seed (0.3 nM) (*47*). The cystatins showed differences in affinity for papain, bromelain, chymopapain and ficin with different inhibitors, showing different spectra of inhibition across the four tested cysteine proteinases. For example, Pap1 showed strong inhibition of papain, chymopapain and ficin, with a significantly lower ability to inhibit bromelain. In contrast, the HMW papain inhibitor, HMWPap2 (assayed as a composite of HMWPap2-1, the minor component, and HMWPap2-2, the major component), showed similar K_Is for each of these enzymes, with higher K_Is for both papain, chymopapain and ficin when compared with Pap1 and Pap2 (assayed as a composite of the minor Pap2-1 and the major Pap2-2 fraction). These K_I values are comparable to those observed for other plant cystatins (*8*, *14*, *23*), but considerably lower than that observed for papain inhibition by a rice cystatin (*20*).

Although the kinetics of inhibition have been measured in plant LMW cystatins (*56*), nothing has been reported on the kinetics for the HMW cystatins. We measured the kinetics of inhibition for the HMWPap2 inhibitor to determine whether this inhibitor shows similar kinetics to other inhibitors. Although this preparation included both HMWPap2-1 and HMWPap2-2, the HMWPap2-2 protein is the major inhibitory species present. Compared to the kinetics of inhibition for a range of animal cysteine proteinases by animal and protozoan cystatins, the k_{ass} of the HMWPap2 inhibitory fraction (1.1×10^6 $M^{-1} \cdot s^{-1}$) is on the lower side of the range of published values (which range up to ~100 times higher), while the k_{dis} (8.1×10^{-5} $M \cdot s^{-1}$) is of the same order as other published values (*41*, *56–58*). This results in a K_I in the sub nM range (0.074×10^{-9} M; lower than that measured by equilibrium methods), which is significantly higher than the K_I measured for the animal cystatins inhibiting animal and protozoan cysteine proteinases. In a kinetic measurement for inhibition of papain by an animal stefin (*41*), the values for k_{ass} are similar to those reported in this study for the HMWPap2 inhibitory fraction, and their k_{dis} values are higher than for the HMWPap2 inhibitory fraction, resulting in higher K_I values for the animal stefin against papain than observed here.

We have found matching genes for some of these inhibitors in the HortResearch EST database, and have used this to identify the apple seed cystatins further based on the predicted peptide sequences. The N-terminal sequence from the purified Pap2-2 inhibitor was identical to an internal predicted peptide sequence from an apple seed EST, as well as to two ESTs from vegetative buds. Using the measured sequence as the start of the protein, we calculated a molecular weight of 11,651.29 for this EST sequence over 103 residues, which compares with a measured mass of 11,633 for Pap2-2. Whether this is due to experimental or calculation (*e.g.*, salt status) error or reflects a mismatch in the amino acid sequence is unknown. This EST showed closest identity with an insoluble carrot seed cell wall cystatin, and both inhibitors showed the three diagnostic motifs of a plant cystatin. The first of these matched the motif perfectly and is located on an alpha helix that is removed from the binding region of cystatins (*53*). The second motif (QVVSG) also matched the consensus sequence (QXVXG) perfectly and is localised at the end of the two beta sheets that form a primary binding site for cystatins, while the sequence at the binding-site junction of the second set of beta sheets (DSEVVVKPW) showed a lower homology to the cystatin motif [EAK(VF)WVKPW; from Ref. *22*].

The purified HMWPap2-2 inhibitor had an internal sequence that perfectly matched 28 ESTs from a range of tissues, but none from seeds. Seeds, at different stages of development, were used in preparing the libraries and so it is surprising that no matching ESTs were identified from this source. However, considering the number of ESTs discovered that putatively correspond to HMWPap2-2, and the high level of this cystatin measured in apple seeds, HMWPap2-2 is obviously a ubiquitous and highly expressed cystatin in apple. This is in contrast to Pap2-2, which was only represented by 3 ESTs. In Genbank, we can discover only 13 sequences that appear to be similar to the HMW cystatins, compared to over 80 clones that fall into the general class of plant cystatins.

By estimating the initial and terminal residues of the EST that putatively corresponds to HMWPap2-2, a molecular mass of 22584 was determined, which compares with a measured mass of 22591 for the purified HMWPap2-2 protein. Again, we are not certain of the reason for this discrepancy, although in this case we cannot be certain as to the end of the presequence. In common with Pap2-2, the first two cystatin motifs perfectly matched the published motif, while the third motif showed some discrepancies.

A Blast search into GeneBank using the homologous HMW Soyacystatin peptide sequence matched 13 predicted peptide sequences over 140 or more amino acids. These included cystatins from *Arabidopsis thaliana* (two sequences), rice, castor bean, sesame, sweet potato (two sequences), tomato, *Brassica rapa* (two sequences),

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est187726F      MATLGGVHESHG------AQNSAEVEDLARFAVQEHNNKENALL--EFVSVVKAKE
est187726B      SSDLGVKQGGHPPGWQSVPPHDPQVQDAANHAVKSLQQRSNSLLPYELQEVVHAQA
                 : **  : .*       . :..:*:* *..**:. ::::.*:**  *: .**:*:

est187726F      QVVAGTLHHLTIEAIEAG-KKKLYQAKVWVKPWMGFKEVQEFKHADEEET
est187726B      EVAEEHAKFNMLLKVKRGSKEEKFKAEVHKN----MEGTFSLNQMEADHS
                :*.    :.  :  :: * *:: ::*:*  :    :: . .::: : :.:

1944319F      MATIGGLRDSQGS------QNSVQTEALARFAVDEHNKKQNSLLEFS--RVVRTQEQVVA
1944319B      SADLGVKKDGHQPGWQSVPTHDPQVQDAANHAIKTIQQRSNSLVPYELHEVADAKAEVID
                  * :*  :*.: .       :. *.:  *..*:.  :::.***: :.  .*. :: :*:

1944319F      GTLHHLTLEAIEAGEKK-LYEAKVWVKPWLNFKELQEFKPAGDVPSFT
1944319B      DFAKFNLLLKVKRGQKEEKFKVEVHKNNQGGF-HLNQMEQDHS-----
                 .  :.  *  :: *:*:  ::.:*  :   .* .*::::   .
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Fig. 9. **Alignment of the two halves of the predicted sequence of the HMW papain inhibitor from apple and soycystatin.** The EST sequence (est187726F and B) represents the front and the back half of the EST which corresponds putatively with HMWPap2-2. The cystatin conserved motifs in the first half of the molecule are highlighted. Similarly, the Soyacystatin front and back halves are labelled 1944319F and 1944319B.

Brassica oleracea, and *Sandersonia aurantiaca*. The cystatins from *Arabidopsis* had full-length (including putative leader sequences) predicted molecular weights of ~26,500 Da compared to the LMW *Arabidopsis* cystatins which had molecular weights in the range of 12,500 to 16,000 Da. In addition, an eight-component multicystatin from potato (*54*) and a three-component multicystatin from sunflower (*23*) also showed sequence homology (data not shown). Ignoring the first *ca.*50 amino acid residues in Soyacystatin (which were not present in all HMW cystatins), Soyacystatin is *ca.*180 amino acid residues long. This is considerably longer than other LMW plant cystatins, which are around 100 amino acid residues long. The sequence of Soyacystatin also matches closely with multicystatin, a potato cystatin that has eight repeating units, each homologous to the basic plant cystatin (*54*) and to a three-domain multicystatin from sunflower (*23*). The first half of Soyacystatin (from residue 50) matches very closely with the multicystatin in each of the eight domains, with the second half of the Soyacystatin molecule displaying reasonable sequence homology to the downstream adjacent multicystatin domain. This suggests that Soyacystatin, and other homologous HMW cystatins, including HMWPap2-2 from apple, comprise two domains, each one a cystatin. Matching the two halves of each of the predicted sequences of apple EST and of the Soyacystatin showed some identity (Fig. 9). For the EST sequence, there were 20 identities out of ~115 residues, with another 32 amino acids showing similarity, while a lower incidence of identity/similarity was observed in the Soyacystatin sequence with values of 17 and 28 respectively out of ~100 residues. In comparison, the three cystatin domains of the sunflower multicystatin show 66 to 88% identity to each other, and the potato domains show 53 to 89% identity. However, the conserved cystatin domains were not apparent in the second half of the Soyacystatin or the apple EST putatively corresponding to HMWPap2-2. This suggests that the HMW cystatins, such as Soyacystatin or HMWPap2-2, were derived by domain doubling, but each half may not have maintained functionality, unlike the multicystatins, which have multiple functional domains (*54*, *55*). The multicystatin from sunflower is probably processed *in vivo* to release three LMW functional cystatins (*23*), but that from potato is not (*55*). It is unlikely that the apple HMW papain inhibitor undergoes processing of this sort, as we only observed the HMW form of this cystatin, and the sequences of the LMW papain inhibitors did not match the HMW form. Thus the HMWPap2-2 inhibitor shows considerably different properties from other cystatins. Not only is it about twice the molecular weight of the conventional cystatins, it has an acidic pI, and apparently two domains, of which probably only one is functional.

For the apple seed trypsin inhibitor, Trp1 we have no corresponding EST sequence to compare predicted amino acid sequences with other inhibitors of the same class. The peptide sequence that was determined suggested that the inhibitor was homologous to proteinase inhibitor 1 (clan 5S according to Ref. *4*). Using an analysis based on amino acid composition, a plot of the percentage amino acid composition for the apple trypsin inhibitor against both the *Amaranthus* and squash inhibitors gave highly significant r^2 values of 0.41 and 0.71 respectively, supporting the classification of the inhibitor as a member of the proteinase 1 clan. Our measured mass of 7,716 is typical of this proteinase inhibitor clan, which is widely spread in many plant families and is found in seeds, tubers and other organs (*4*). However, a Blast search using the full sequences from *Amaranthus* and squash revealed no members of the proteinase inhibitor 1 clan among the apple EST database.

No sequence data was observed for Pap1, probably because the protein was N-terminally blocked. However,

comparative analysis of the amino acid composition showed some similarity to the Pap2-2 amino acid composition (r^2 = 0.68) and carrot cell wall cystatin (r^2 = 0.44) but little to the cysteine proteinase inhibitor b from sunflower seeds (r^2 = –0.13). On the other hand, the Pap2-2 amino acid content correlated well with the homologous apple EST (r^2 = 0.97), the carrot cell wall cystatin (r^2 = 0.56), and the sunflower cystatin (r^2 = 0.29). We are proposing that Pap1 is a cystatin because of its ability to inhibit papain, its molecular weight, and because of its amino acid composition similarity to Pap2-2, an identified cystatin. However, Pap1 appears to be significantly different from Pap2-2 and, although a cystatin, it represents a different group of cystatins from the Pap2-2 group. Searches through Genbank for protease inhibitors revealed that sequence of only one trypsin inhibitor (from *Ficus carica*) and one cysteine protease inhibitor (from *Pyrus communis*) have been deposited in this database from species belonging to the Rosales, an important and diverse group of plants. Neither of these sequences appears to have been characterised biochemically.

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