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Purification and characterization of phytocystatins from kiwifruit cortex and seeds☆

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Abstract

Kiwifruit cysteine proteinase inhibitors (KCPIs) were purified from the cortex and seeds of kiwifruit after inactivation of the abundant cortex cysteine proteinase actinidain. One major (KCPI1) and four minor cystatins were identified from *Actinidia deliciosa* ripe mature kiwifruit cortex as well as a seed KCPI from *A. chinensis*. The predominant cortex cystatin, KCPI1, inhibited clan CA, family C1 (papain family) cysteine proteinases (papain, chymopapain, bromelain, ficin, human cathepsins B, H and L, actinidain and the house dust mite endopeptidase 1), while cysteine proteinases belonging to other families, [clostripain (C11), streptopain (C10) and calpain (C2)] were not inhibited. Inhibition constants (K_I) ranged between 0.001 nM for cathepsin L and 0.98 nM for endopeptidase 1. The K_I (14 nM) for KCPI1 inhibiting actinidain is at least 2 orders of magnitude higher than for other plant proteinases measured. The cortex KCPI1 and a seed KCPI purified from seeds had the same N-terminal sequence (VAAGGWRPIESLNSAEVQDV). BLAST-matching the peptide sequence against an in-house generated *Actinidia* EST database, identified 81 cDNAs that exactly matched the measured KCPI1 peptide sequence. Peptide sequences of two other cortex KCPIs each exactly matched a predicted peptide sequence of a cDNA from kiwifruit. The predicted peptide sequence of KCPI1 of 116 amino acids encodes a signal peptide and does not contain cysteine. Without the signal peptide (mature protein), KCPI1 has a molecular mass of ~11 kDa, possesses the consensus sequence characteristic for the phytocystatins and shows the highest homology to a cystatin from *Citrus×paradisi* (52% identity). This is the first report of phytocystatins from the Ericales.

Keywords: *Actinidia deliciosa*; *Actinidia chinensis*; Actinidiaceae; Liquid chromatography; Kinetic constants; Bioinformatics; Cystatin; Actinidain; Seeds; Cortex

1. Introduction

The cystatin superfamily of tight and reversibly binding inhibitors of the papain-like cysteine proteinases (clan CA, family C1) have been divided into three animal families, namely the stefins, the cystatins and the kinenogens, and one plant cystatin family (Abrahamson, 1994). It has been shown that members of this superfamily interact directly with the active site cleft of papain at three regions of the mature cystatin. These are an N-terminal region with a conserved glycine residue, a central loop containing the highly conserved Q-X-V-X-G motif and a C-terminal region with a conserved tryptophan residue (Corr-Menguy et al., 2002). The plant cystatins or phytocystatins are further identified by the consensus sequence (LVI)-(AGT)-(RKE)-(FY)-(AS)-(VI)-X-(EDQV)-(HYFQ)-N found within a predicted amino-terminal alpha-helix (Margis et al., 1998).

Phytocystatins resemble the stefins in lacking disulfide bonds and carbohydrates and having a molecular mass of ~11 kDa, but show greatest amino acid sequence homology to the cystatins (Laing and McManus, 2002). They are abundant in plants and have been identified in seeds, leaves, roots and fruit (Abe et al., 1987; Flores et al., 2001; Gaddour et al., 2001; Pernas et al., 2000a; Ryan et al., 1998; Song et al., 1995). While most commonly they are small proteins ranging from 11 to 13 kDa (Laing and McManus, 2002), a few cystatins are

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Nomenclature

CP	cysteine proteinase
CPI	cysteine proteinase inhibitor
KCPI	Kiwifruit cysteine proteinase inhibitor
DMF	dimethylformamide
E-64	L-*trans*-epoxysuccinyl-leucyl-amido-(4-guanidino)butane
EST	expressed sequence tag
K_I	dissociation constant
k_{ass}	association rate constant
k_{diss}	dissociation rate constant

significantly larger (Joshi et al., 1998; Misaka et al., 1996; Siqueira-Junior et al., 2002).

Plant cystatins are thought to have several possible functions, including regulating the activity of endogenous cysteine proteinases during different physiological processes including seed maturation and germination, and programmed cell death (Arai et al., 2002; Corr-Menguy et al., 2002; Solomon et al., 1999), and acting as plant defensive proteins to biotic and abiotic stresses in a variety of plants (Botella et al., 1996; Cowgill et al., 2002; Gaddour et al., 2001; Gutierrez-Campos et al., 1999; Irie et al., 1996; Joshi et al., 1998; Koiwa et al., 2000; Pernas et al., 1998, 2000a; Siqueira-Junior et al., 2002; Urwin et al., 1997; Walker et al., 1999). Consequently there has been increasing interest in constructing transgenic plants, expressing phytocystatins, resistant to a variety of pests (Gutierrez-Campos et al., 1999; Irie et al., 1996; Michaud et al., 1993; Urwin et al., 1997).

In this work, we report the purification of cystatins from kiwifruit cortex (rich in the cysteine proteinase actinidain) and from seeds, the identification of some of their corresponding genes and kinetic characterization of the predominant cystatin. To our knowledge, this is the first report of the purification and sequencing of a phytocystatin from plant tissue rich in cysteine proteinases. This is also the first report of sequence of a proteinase inhibitor, let alone phytocystatin, to be deposited in GenBank from the order Ericales, which includes the Actinidiaceae, Balsaminaceae, Ebenaceae, Lecythidaceae, Primulaceae, Polymoniaceae and Theaceae families.

2. Results

2.1. Purification of cysteine proteinase inhibitors

Kiwifruit cortex has high levels of a cysteine proteinase called actinidain (Praekelt et al., 1988), which may bind and mask, or degrade, endogenous CPIs present in the tissue. In addition, CPs can bind cystatins even when the active site cysteine moiety is modified and the enzyme is inactive (Abrahamson, 1994; Lindahl et al., 1992). To avoid losing CPIs in our extract through binding to CPs, we denatured actinidain by heating the extract, pH 3, at 60 °C for 10 min before purifying the inhibitor. Total KCPIs in fresh fruit cortex extract were 7.13 (S.D. = 1.04, $n = 4$) μg/g fresh weight, and the amount of inhibitor was unchanged after one-year fruit storage in air at 0 °C.

In a preliminary experiment, no inhibitory activity towards pepsin, trypsin or chymotrypsin was detected in the cortex extract while apparent pepsin inhibitory activity was detected in the seed extract (data not shown).

Cystatins eluted from the papain affinity column were separated on a HiTrap SP column (Fig. 1) into a major (SP1) and three other minor papain inhibitory peaks (SP2-4). These four peaks were separately pooled and purified by HPLC on a Vydac C-18 column. The elution of the main inhibitory (SP1) peak is shown in Fig. 2. Silver-stained gels of this main inhibitor showed one protein band (KCPI1) with a Mr ~11 kDa (Fig. 3). Similarly reverse-phase chromatography of fraction SP4 resulted in the isolation of different protein bands (KCPI2, KCPI3) from two adjacent inhibitory HPLC fractions (Fig. 3) while SP2 and SP3 protein concentrations were very low and their inhibitory activities were not investigated further.

We also purified a phytocystatin from kiwifruit seeds. Fig. 4 shows the elution from a Vydac C-18 column, of the purified main seed KCPI.

2.2. Inhibition profile

KCPI1 was found to inhibit all cysteine proteinases we tested that belong to clan CA family C1, the papain family of cysteine proteinases (Table 1), except human bleomycin hydrolase. The K_I values ranged between 0.001 nM for human cathepsin L and 98 nM for the house dust mite endopeptidase 1 (Der p 1). Cysteine proteinases belonging to other families, namely clostripain (C11), streptopain (C10) and calpain (C2) were not inhibited at inhibitor/enzyme molar ratios of at least 5/1. We did not measure inhibition by other KCPIs as there was insufficient material available.

2.3. Rate constants of inhibition by KCPI

The rate constants (k_{ass} and k_{diss}) for papain inhibition by KCPI1 were measured by fitting an equation to the time course of the reaction. The fitted value of k_{ass} was $1.60E+06 \pm 2.10E+05$ M^{-1} s^{-1} and k_{diss} was $1.66E-04 \pm 1.18E-04$ s^{-1} giving a calculated K_I of 0.10 nM, which is very close to that determined by the equilibrium method (Table 1).

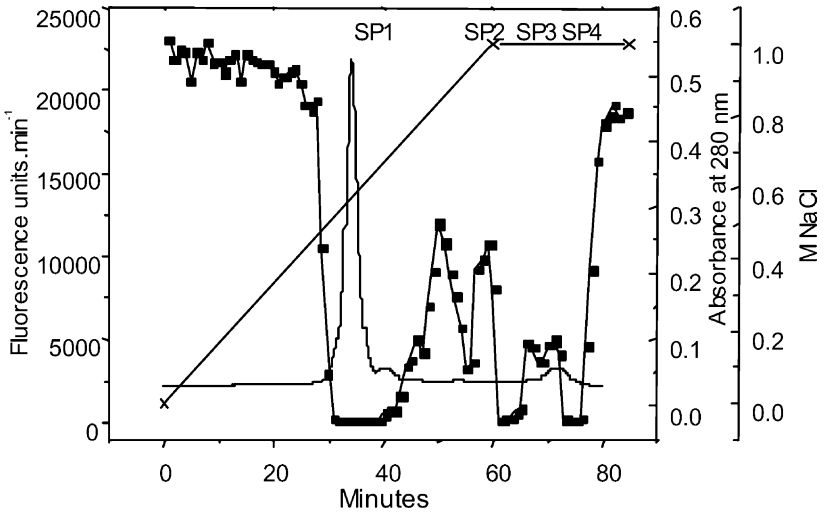

Fig. 1. HiTrap SP column fractions of cortex KCPIs. Protein profile (—) and papain inhibition (■). The column was equilibrated with 50 mM sodium formate pH 4.0; bound proteins were eluted with 1 M NaCl gradient (×—×). Fractions were assayed for papain inhibitory activity using Z-Arg-AMC as substrate. Four inhibitory peaks were observed (SP1-4).

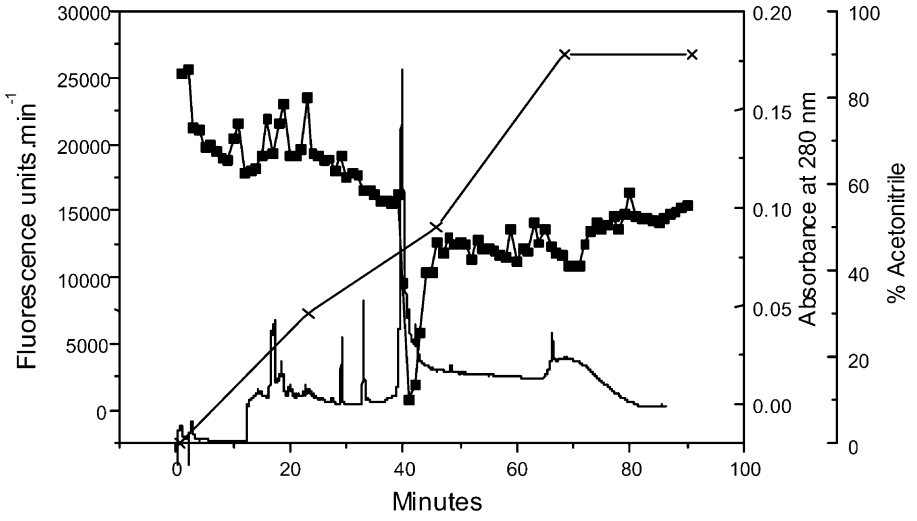

Fig. 2. Vydac C-18 HPLC of KCPI1 (SP1 of Fig. 1). Bound proteins (—) were eluted with acetonitrile gradient in 0.1% TFA (×—×). Fractions were tested, with papain, for inhibitory activity (■) using Ala-Leu-Lys-AMC as substrate.

Table 1
Inhibition of various cysteine proteinases clan CA family C1 by KCPI1

Enzyme	$K_I \pm$S.E. (nM)
Human cathepsin L	0.001±0.0003
Ficin	0.032±0.009
Human cathepsin H	0.049±0.021
Bromelain	0.15±0.04
Chymopapain	0.15±0.04
Papain	0.16±0.04
	0.10[a]±0.09
Human cathepsin B	8.0±0.8
Actinidain	14±2
Endopeptidase 1 (Der p 1)	98±17

Assays were carried out using pre-incubation to equilibrium as described in the methods.

[a] Calculated, from kinetics study, as k_{diss}/k_{ass}.

2.4. Lack of cleavage of KCPI1 by cysteine proteinases

To measure whether a range of papain-like cysteine proteinases were able to cleave the inhibitor, KCPI1 was incubated with at least an equimolar amount of the proteinase for up to 5 h before treatment with sample buffer for SDS–PAGE electrophoresis. Enzymes tested were: papain, actinidain, bromelain, ficin and human cathepsin B (Table 2). No degradation of the cystatin occurred when E-64 was added at the end of incubation period of enzyme with KCPI1 prior to heating in sample buffer, although some digestion occurred when E64 was left out. It is likely that during the heat-denaturing step in sample buffer, the proteinase inhibitor may denature and become vulnerable to degradation by remaining active proteinase. Papain was noted to undergo auto-catalytic degradation, which was abolished by adding

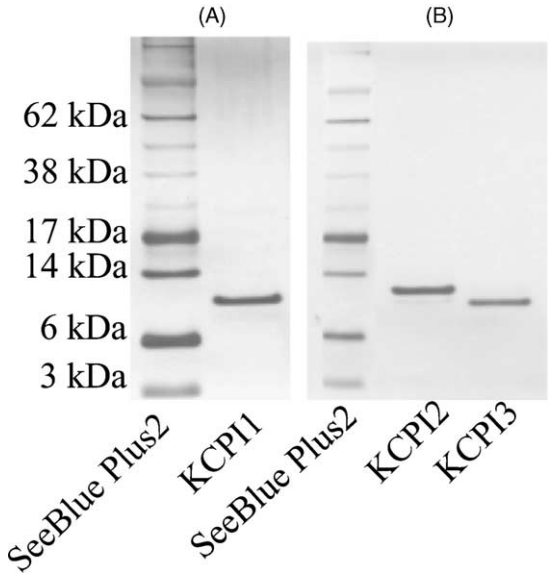

Fig. 3. SDS–polyacrylamide gel electrophoresis of KCPI1 (A) and KCPI2 and KCPI3 (B) under reducing conditions. The NuPAGE™ 12% bis-tris gels (Invitrogen) were run in MES-SDS buffer and silver-stained.

E-64 prior to heating in sample buffer. Thus KCPI1, as has been observed for other cystatins (Lindahl et al., 1992), is not degraded by the cysteine proteinases that it inhibits.

2.5. MALDI–TOF mass spectrometry and sequence analysis

Mass spectrometry of *A. deliciosa* cortex KCPI1 revealed a major peak of 10.928 kDa and a shoulder of 11.087 kDa (Fig. 5). Only a single N-terminal peptide sequence was obtained from this fraction, indicating that either they represent the same molecular species with one either modified or shortened at the C-terminus or else one of them has a blocked N-terminus. Likewise KCPI2 and KCPI3 yielded major protein peaks at ~11.6 and 11 kDa respectively with slightly higher MW shoulders (~11.8 and 11.6 respectively). N-terminal sequencing however, revealed two sequences each (Table 3). *A. chinensis* seed KCPI had a mass of 10.926 kDa and a shoulder at 11.047 kDa (data not shown). Both of these figures are very close to those obtained for the cortex KCPI1 and the N-terminal sequence obtained exactly matched that for KCPI1 (Table 3).

KCPI2 sequence 1 and KCPI3 sequence 1 possessed three extra amino acids upstream of the sequence GGWRPI found in all sequences (Table 3). This could be due to alternative peptidase cleavage or degradation of the N-terminal amino acids of the phytocystatin of the different observed phytocystatin genes.

BLAST (Altschul et al., 1997) searches using these sequences to find matches in the HortResearch *Actinidia* EST database, containing over 92,000 sequences from 36 EST libraries, revealed exact matches to three out of the five measured sequences and partial matches to the other two (KCPI 3 sequences 1 and 2). The measured KCPI1 peptide sequence exactly matched 81 ESTs from *Actinidia*. Seventy-seven ESTs were from *A. deliciosa* ripe fruit inner cortex, two from *A. deliciosa* petal library and the other two from *A. chinensis* ripe fruit. The 20 amino acid sequence of KCPI1 matched to amino acids 27–46 of the predicted cystatin amino acid sequence (Fig. 6). The computed theoretical MW for KCPI1 sequence including the signal peptide is 12.756 kDa, with a calculated pI of 9.4. The corresponding MW for the mature phytocystatin is 10.053 kDa, (with a pI of 6.91), which is at least 0.875 kDa less than that

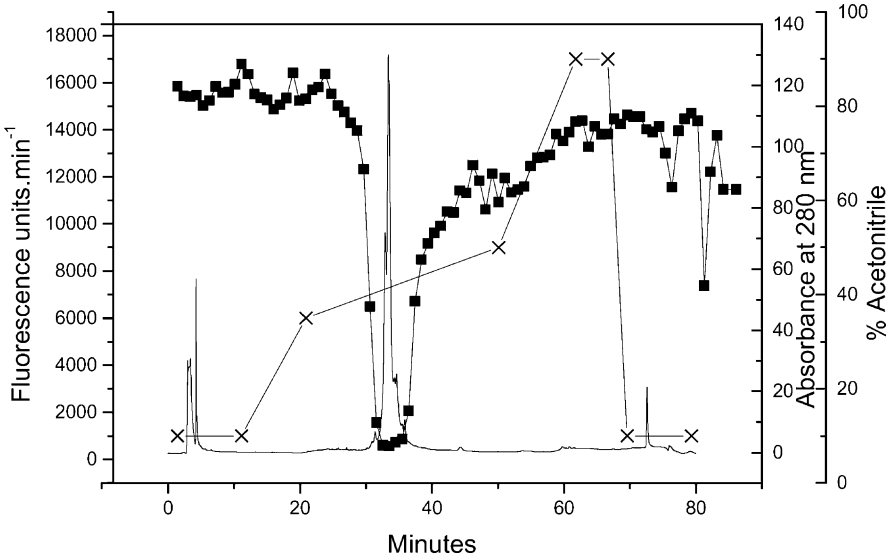

Fig. 4. Elution profile of the main seed KCPI from Vydac C-18 column. Bound proteins (—) were eluted with acetonitrile gradient in 0.1% TFA (×—×). Fractions were tested with papain for inhibitory activity (■) using Ala-Leu-Lys-AMC as substrate.

Table 2
Enzymes, substrates and assay buffers used for inhibition studies

Enzyme (activation)	Proteinase per well (ng)	Source	Assay buffer[a]	Substrate
Actinidain EC 3.4.22.14 (10 min in assay buffer)	5–100	Purified in the lab.	0.1 M Bis-tris- propane (or MOPS), 2 mM EDTA, 2 mM DTT, pH 7.0	H-D-Ala-Leu-Lys-AMC[b]
Bromelain EC 3.4.22.4 (10 min in assay buffer)	100–500[e]	Sigma	0.1 M MOPS, 2 mM DTT, 2 mM EDTA, pH 7.0	H-D-Ala-Leu-Lys-AMC[b]
Calpain, EC 3.4.22.17 (10 min in assay buffer)	50–500	Sigma	0.1 M Bis-tris-propane, 2 mM EDTA, 2 mM DTT, pH 7.0	Suc-Leu-leu-Val-Tyr-AMC[b]
Human Cathepsin B EC 3.4.22.1 (10 min in assay buffer)	1–10	Athens Research & Technology, Inc.Georgia	0.1 M MES, 2 mM DTT, 2 mM EDTA, pH 6.0	Z-Arg-Arg-AMC[b]
Human Cathepsin H EC 3.4.22.16 (10 min in assay buffer)	125	Athens Research & Technology, Inc.Georgia	0.075 M K phosphate, 1 mM EDTA, 3 mM cysteine, pH 6.8	H-Arg-AMC[b]
Human Cathepsin L EC 3.4.22.15 (10 min in assay buffer)	1–10	Athens Research & Technology, Inc. Georgia	0.4 mM Na acetate buffer, 4 mM EDTA, 8 mM DTT, pH 5.5	Z-Phe-Arg-AMC[b]
Chymopapain EC 3.4.22.6 (10 min in assay buffer)	100–500[e]	Sigma	0.1 M MOPS, 2 mM DTT, 2 mM EDTA, pH 7.0	H-Pro-Phe-Arg-AMC[b]
Chymotrypsin EC 3.4.22.1 (none)	1–10[e]	Sigma	0.1 M Tris, 2 mM $CaCl_2$, pH 8.0	Suc-Ala-Ala-Pro-Phe-AMC[b]
Clostripain, EC 3.4.22.8 (2–3 h in assay buffer)	200–800	Sigma	0.1 M MOPS, 1 mM $CaCl_2$, 2.5 mM DTT, pH 7.4	Z-Phe-Arg-AMC[b]
Endopeptidase 1 (Der p 1) (10 min in assay buffer)	50–250	F Shakib Queen's Medical Centre, Nottingham, UK	0.05 mM K phosphate pH 8.25 OR 0.1 M Na phosphate pH 6.5, 5 mM cysteine or DTT	N-t-Boc-Gln-Ala-Arg-AMC[c]
Ficin EC 3.4.22.3 (10 min in assay buffer)	6–60[e]	Sigma	0.1 M MOPS, 2 mM DTT, 2 mM EDTA, pH 7.0	H-D-Ala-Leu-Lys-AMC[b]
Papain EC 3.4.22.2 (10 min in assay buffer)	10–100[e]	Sigma	0.1 M MOPS, 2 mM DTT, 2 mM EDTA, pH 7.0	H-D-Ala-Leu-Lys-AMC[b] or Z-Arg-AMC[b]
Pepsin EC 3.4.23.1 (none)	20–200[e]	Sigma	0.75% lactic acid/HCl, pH 2.0	Bodipy-casein (EnzChek™ protease assay kit green fluorescence[d]
Streptopain EC 3.4.22.10 (10 min in assay buffer)	20–200	G. Card and H. Baker, School of Biological Sciences, Auckland	0.1 M Na phosphate, 10 mM cysteine, pH 7.0	Bz-Phe-Val-Arg-AMC[b]
Trypsin EC 3.4.21.4 (none)	10–100[e]	Sigma	0.1 M Tris, 2 mM $CaCl_2$, pH 8.0	Z-R-7-amido-4-methylcoumarin (Z-Arg-AMC)[b]
Human Bleomycin Hydrolase EC 3.4.22.40 (10 min in assay buffer)	200	R. Koldamova, University of Pittsburgh, Pittsburgh	0.1 M MOPS, 10 mM DTT, pH 7.2	H-Citrulline-AMC[b]

[a] All assays contained Tween-20 at a final concentration of 0.025 μl/ml.
[b] BACHEM Feinchemikalien AG, Bubendorf, Switzerland.
[c] Sigma.
[d] Molecular Probes, Inc.
[e] Determined from weight measurement.

experimentally determined by MALDI–TOF. This could be due to post-translational modification, for example glycosylation. However, no carbohydrate moiety was detected in KCPI1 using a DIG Glycan Detection Kit (Roche Diagnostics). Other possibilities including formylation, acetylation, phosphorylation & methylation though cannot be excluded, seem to be unlikely to account for the calculated large difference between experimentally determined and calculated mass of this soluble protein.

KCPI2 sequence 1 gave a perfect match to an EST from *A. deliciosa* ripe fruit inner cortex while sequence 2 gave a perfect match to two ESTs found in *A. eriantha* young fruit (Fig. 6). In these cDNAs, a pre-sequence of 22 and 25 amino acids respectively, starting with methionine, preceded the sequenced amino acids. KCPI2 sequences 1 and 2 have predicted MWs of 12.9 and 12.7 kDa with theoretical PIs of 10.06 and 9.4 respectively. The predicted mature protein MWs, are 10.7 and 10.1 kDa with PIs of 9.99 and 6.91 respectively.

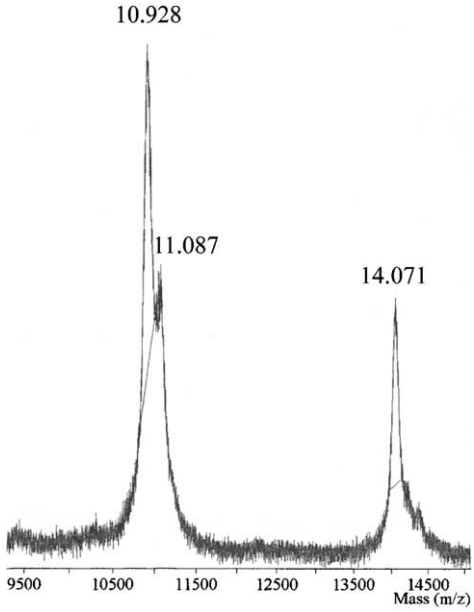

Fig. 5. MALDI–TOF mass spectrometry of KCPI1 showing a main peak of 10.928 kDa and a shoulder of 11.087 kDa relative to the calibrant, α-lactalbumin (14.071 kDa).

The aligned predicted amino acid sequences of full length ESTs that exactly matched our three CPI peptide sequences (Fig. 6), have glycine at the extremity of the N-terminal region within the mature protein and the consensus V-A-Q-F-A-V-S-E-H-N at the alpha1 helix terminal, which differs from that for the phytocystatin consensus sequence, (LVI)-(AGT)-(RKE)-(FY)-(AS)-(VI)-X-(EDQV)-(HYFQ)-N), only at the third position where Q replaces (RKE). Q-X-V-X-G is present in the first binding loop characterized by this motif. The second less conserved binding loop (ED)-A-V-(VF)-W-D-K-P-W) is similar to the phytocystatin consensus E-A-K-(VF)-W-V-K-P-W (Margis et al., 1998).

KCPI3 sequence 1 had no exact matches to the EST database, but did show identity at the level of 16 to 17 out of 20 residues to ESTs, encoding cystatins, from *A. deliciosa* ripe fruit inner cortex and from dormant kiwifruit buds. KCPI3 sequence 2 (Table 3) also showed 17

Table 3
N-terminal sequences of KCPI1, KCPI2 and KCPI3

Inhibitor	Variant sequence	Sequence
KCPI1		VAAGGWRPIESLNSAEVQDV
Seed KCPI		VAAGGWRPIESLNSAEVQDV
KCPI2	SEQUENCE 1	RKQVVLGGWRPIKDLNSAEVQD
	SEQUENCE 2	VAPGGWRPIENLNSAEVQDVAQ
KCPI3	SEQUENCE 1	VKQVVLGGWRPIKDLNVADV
	SEQUENCE 2	RAAGGWRPIESLNSAESQEV

The shaded boxes represent conserved sequences.

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103292 (KCPI1)    MVPKPLSLLLFLLLALSAAVVGGRKLVAAGGWRPIESLNSAEVQDVAQFA
101993 (KCPI2-1)  MVPKTFS-MLLLLIALSAAVVGGRKQVVLGGWRPIKDLNSAEVQDVAQFA
166750 (KCPI2-2)  MVPKPLSLL-FLLLALSAAVVGGRKLVAPGGWRPIENLNSAEVQDVAQFA
AAG38521          MNQRFCCLIVLFLSVVPLLAAGDRKGALVGGWKPIEDPKEKHVMEIGQFA

103292            VSEHNKQANDELQYQSVVRGYTQVVAGTNYRLVIAAKDGAVVGNYEAVVW
101993            VSEHNKQANDKLQYQRVVRGYSQVVAGTNYRLVIAAKDGAVLGKYEAFVW
166750            VSEHNKQANDELQYQSVVRGYTQVVSGTNYRLVIAAKDGAVVGNYEAVVW
AAG38521          VTEYNKQSKSALKFESVEKGETQVVSGTNYRLILVVKDGPSTKKFEAVVW

103292            DKPWMHFRNLTSFRKV*
101993            DKPWMQFRNLTSFRKV*
166750            DKPWMHFRNLTSFRKV*
AAG38521          EKPWEHFKSLTSFKPMVK*
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Fig. 6. CLUSTAL X multiple sequence alignment of predicted amino acid sequences deduced from cDNAs encoding cystatin sequences that are identical to the N-terminal sequences obtained from kiwifruit. AAG38521 is a *Citrus×paradisi* Genbank cystatin sequence showing the most identity to the measured KCPI sequences. Bold characters represent the measured peptide, shaded characters represent identical sequences.

out of 20 amino acid identity to a range of *A. deliciosa* inner fruit cortex and petal predicted cystatin sequences.

GenBank BLAST searches (Altschul et al., 1997) showed that all five KCPIs are most similar to a cystatin-like protein from *Citrus×paradisi* (45–54% identity) (Fig. 6) and *Arabidopsis thaliana* (57–46% identity). Taxonomically, *Actinidia deliciosa, Citrus×paradisi* and *Arabidopsis thaliana* are core eudicots. *Actinidia deliciosa* is more remotely related to *Coix lacryma-jobi, Oryza sativa*, *Sorghum bicolor* and *Zea mays*, which possess cystatins with identities ranging between 46 and 51%. While a number of cystatins have been reported from Asteridae, notably: potato (accession number Q03196), tomato (A59155), sunflower (JC7333), carrot (T14323), short ragweed (JN0906), sweet potato (AAF64480) and sesame (AAK15090), no cystatin has yet been identified in the Ericales.

CLUSTAL analysis (Jeanmougin et al., 1998) of a selection of apple and kiwifruit cystatin translated full length cDNA sequences from the HortResearch EST database along with eight *Arabidopsis* putative cystatin translated gene sequences (Laing and McManus, 2002) showed that the cystatins fell into three broad clusters, each with apple, kiwifruit and *Arabidopsis* sequences (Fig. 7).

3. Discussion

There are at least five phytocystatin protein sequences in kiwifruit (Table 3), with three matching identified gene sequences (Fig. 6), and several other cystatin variants were also observed in the EST database from kiwifruit (Fig. 7), which may represent further cystatins. The cDNA for KCPI1 was extremely well represented in the kiwifruit inner cortex library, consistent with the higher level of expression of this protein compared to KCPI2 and KCPI3. As the fruit inner cortex used to

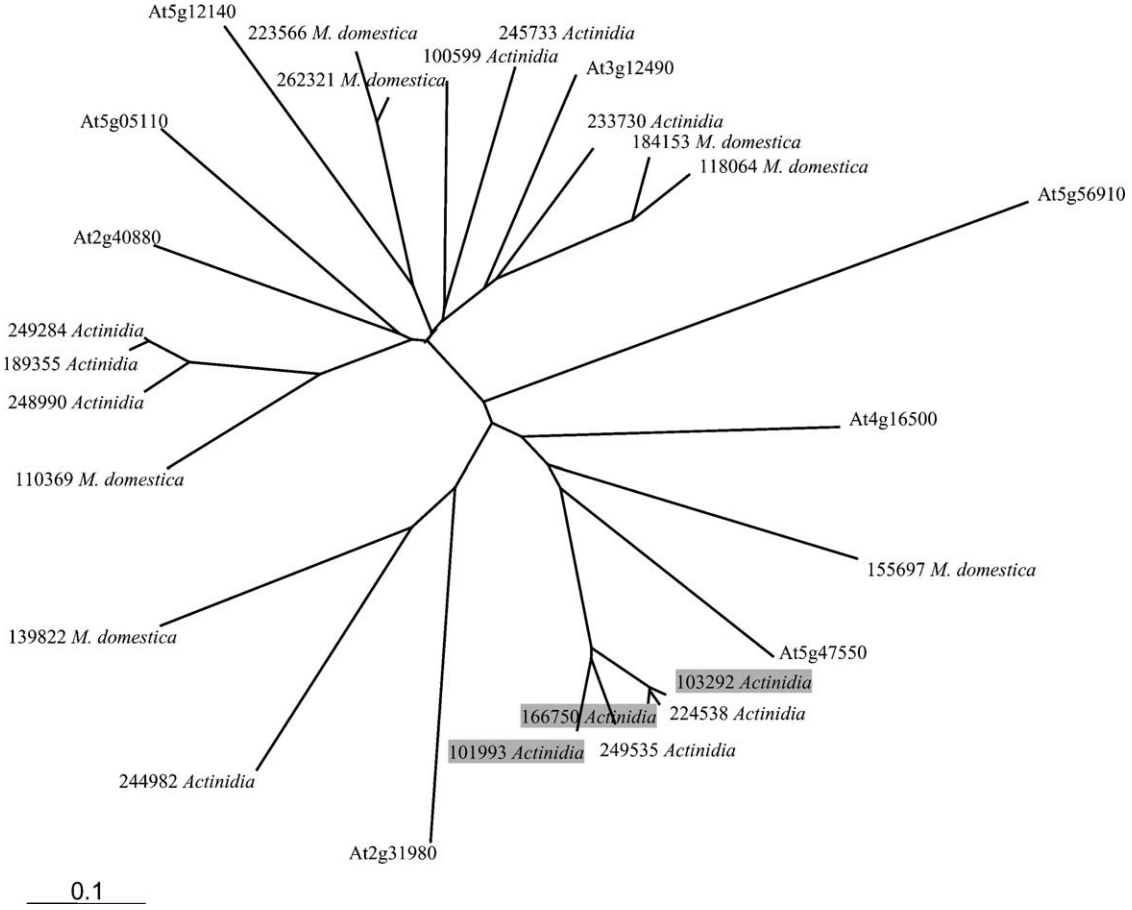

Fig. 7. Cluster analysis of cystatins from kiwifruit and apple with *Arabidopsis* cystatins. Full length cystatin sequences were clustered using CLUSTAL X. Cystatins labelled *Actinidia* come from either *A. deliciosa* ripe fruit inner cortex (100599, 101993, 103292), *A. eriantha* young fruit (166750), *A. arguta* petal (248990, 249535), *A. eriantha* petal (224538), *A. chinensis* petal (245733, 249284), *A. chinensis* ripe fruit (189355, 244982) or dormant *A. deliciosa* buds three days after hydrogen cyanamide treatment (233730). Apple (*Malus domestica*) sequences included spur buds from Pacific Rose trees (155697), Royal Gala fruit stored for 24 h under low oxygen/high CO_2 (118064), Royal Gala phloem (118064), Royal Gala partially senescing leaf (223566), *Malus domestica* cell culture (262321), Royal Gala 10 days after full bloom fruit (110369) and Royal Gala pre-opened vegetative bud (139822). At numbers refer to *Arabidopsis thaliana* genome sequences from the MIPs database (http://mips.gsf.de/proj/thal/db/). Shaded EST names are the sequences shown in Fig. 6.

make the cDNA libraries included seeds, it is possible that some of the observed sequences of KCPI come from the seeds. The genome of *Arabidopsis* contains at least eight phytocystatin genes with a predicted molecular weight consistent with the kiwifruit phytocystatins (Laing and McManus, 2002). We chose to do cluster analysis of the kiwifruit cystatins in relation to those predicted in *Arabidopsis* as the complete genome of *Arabidopsis* is known, giving us a full spectrum of a dicotyledonous species' cystatins. We also included predicted apple cystatins that have also been discovered in HortResearch EST database. The purified cystatins described in this paper all fell in one group (Fig. 7), which included the nearest hits in the EST database to KCPI3-1 and -2, which did not have exact matches. Kiwifruit cystatin ESTs were found in all three clusters, as were petal ESTs. Except for the clustering of the EST cystatins purified in this paper, there appeared to be little other functional clustering of cystatins.

Part or all of the first 26, 22 or 25 amino acid sequence of KCPI1, KCPI2 sequences 1 or 2 respectively, preceding the sequenced amino acids, are predicted to be a signal peptide involved in attaching the nascent polypeptide to the membrane for transport to non-cytoplasmic locations or for secretion to the extracellular fluid (Briggs and Gierasch, 1986). A signal peptide has been identified for oryzacystatin-1 (Womack et al., 2000) and is suggested to direct the pre-protein to the endoplasmic reticulum for processing to the mature form. Sunflower cystatin Scb is synthesised as a pre-peptide consisting of a 22 amino acid signal sequence and a peptide of 101 amino acids for the mature protein (Doi-Kawano et al., 1998). An 18 kDa extracellular insoluble cystatin of carrot (Ojima et al., 1997) was found to consist of 133 amino acid residues that included a signal sequence.

KCPI1 was found to be a very potent inhibitor of those papain-like cysteine proteinases, from human, animal or plant origin, we tested. Human cathepsin L was extremely susceptible to inhibition by KCPI1 and to the best of our knowledge no such powerful inhibitory activity has been reported for phytocystatins (Abe et al., 1994; Rogelj et al., 1998). The sensitivity of the cathepsins towards KCPI1 was in the order: cathepsin L > cathepsin H > cathepsin B, similar to that observed for bovine stefin A (Turk et al., 1995), while corn cystatin 1 was far more inhibitory to cathepsin H and showed little ability to inhibit cathepsin B (Abe et al., 1994). The kiwifruit cystatin is more than two orders of magnitude more effective an inhibitor of human cathepsin H than chelidocystatin (Rogelj et al., 1998), corn cystatin 1 (Abe et al., 1994) and oryzacystatin-II (Kondo et al., 1990). KCPI1 is also about 60× and 40× more inhibitory to cathepsin B than chestnut cystatin (Pernas et al., 1998) and corn cystatin 1 (Abe et al., 1994).

The K_I for the inhibition of papain by KCPI1 (0.16 nM) is of the same order of magnitude as that of papain inhibition by chelidocystatin (Rogelj et al., 1998) and by a cysteine proteinase inhibitor from apple fruit (Ryan et al., 1998). Oryzacystatin-1 (Kondo et al., 1990), corn cystatin 1 (Abe et al., 1994), a recombinant barley CPI (Gaddour et al., 2001) and a chestnut seed cystatin however (Pernas et al., 1998) are more than two orders of magnitude less inhibitory to papain than KCPI1. KCPI1 is also two to three orders of magnitude more inhibitory to ficin than barley (Gaddour et al., 2001) and chestnut cystatin (Pernas et al., 1998). The house dust mite, *Dermatophagoides pteronyssinus,* endopeptidase 1 (Der p 1) was inhibited by KCPI1 while it was not inhibited by the chestnut cystatin which was only inhibitory to the house dust mite *Dermatophagoides farinae endopeptidase* 1 (Der f 1) (Pernas et al., 2000b). In contrast to the chestnut cystatin (Pernas et al., 1998), KCPI1 possessed no inhibitory properties towards serine proteinases.

While KCPI was effective against a range of plant and non-plant cysteine proteinases belonging to clan CA family C1, it was not as effective against actinidain ($K_I = 14$ nM), the main endogenous cysteine proteinase in kiwifruit cortex. Such relatively low affinity towards actinidain was also observed for recombinant human cystatin C (Bjork et al., 1994) and chicken egg white cystatin (Bjork and Ylinenjarvi, 1990). Actinidain was about two orders of magnitude less sensitive to inhibition by KCPI1 than papain, bromelain and chymopapain. KCPI1 did not inhibit cysteine proteinases (calpain, clostripain, streptopain and bleomycin hydrolase) little affected by E-64. Interestingly, bleomycin hydrolase, the only member of clan CA family C1 that was not inhibited by KCPI1, is also relatively insensitive to E-64 (Bromme et al., 1996) compared to other cysteine proteinases (Barrett et al., 1982).

There is very limited published kinetic analysis of the interaction between phytocystatins and papain-like proteinases, but much more for the animal cystatins. In the case of an apple high molecular weight phytocystatin and papain (Ryan et al., 2003), the rate constants for association and dissociation (k_{ass} and k_{diss}) are similar to those measured for the kiwifruit phytocystatin in this paper. The k_{ass} for the inhibition of papain by KCPI1, is also similar to that found for bovine stefin C when inhibiting papain (Turk et al., 1993) and is almost an order of magnitude lower than that of chicken and recombinant human cystatin C and papain (Bjork et al., 1994; Lindahl et al., 1992). The k_{diss} however, is at least half of that measured for stefins and papain (Turk et al., 1993) and more than 1000× higher than that for human cystatin C and papain (Lindahl et al., 1992). This explains why chicken and human cystatin C (Bjork et al., 1994) are much better inhibitors of papain than KCPI1.

The function of this cystatin in the cortex is presumably not to protect the cell against actinidain as firstly, actinidain is present in vast excess, and secondly it is not a particularly effective actinidain inhibitor. Perhaps it functions to control minor endogenous cysteine proteinases, or it is a plant defence protein against insect/pathogen attack. This latter function may be more feasible for seeds where actinidain is absent while the vast excess of actinidain over cystatin in the cortex may prevent the inhibitor from functioning on exogenous proteinases after the cells are disrupted.

In summary, we have reported the presence of a family of phytocystatins in kiwifruit despite the presence of high levels of the cysteine proteinase, actinidain, which is inhibited by the most predominant of the KCPIs. KCPI1, like the animal stefins and cystatins, inhibits papain-like cysteine proteinases of plant, animal or human origin while it is superior to other phytocystatins in its inhibitory activity towards human cathepsin L.

4. Materials and methods

4.1. Plant material

Mature fresh fruits from *Actinidia deliciosa* (A. Chev.) C. F. Liang *et* A.R. Ferguson var. deliciosa 'Hayward', cv. Hayward, stored in air at 0 °C, were used throughout the present work for cortex extraction. Kiwifruit seeds were obtained from ripe fruit of *Actinidia chinensis* Planch. var. chinensis 'Hort16A'.

4.2. Seed preparation

One hundred mature fruits were cut in half, the flesh scooped out and incubated in 5 l of water with 1 ml of diluted (1:200, v/v) pectalase (Rohapect, D5L special pectalase, Carter Associates). The suspension was left at 20–25 °C for 2–3 days and the pulp was then washed through a sieve, using a jet of water, leaving the seeds behind. These were blotted-dry, frozen and ground in a cryo-mill, at liquid nitrogen temperatures, to a fine powder.

4.3. Papain affinity chromatography

A 10 ml papain affinity column with either active (i.e. in the presence of DTT (Ryan et al., 1998)) or inactive papain (inactivated by carboxy-methylation of the sulfhydryl group with iodoacetamide (Brocklehurst et al., 1981) was used to purify the CPIs with similar results. The columns were eluted (1 ml/min) with 20 mM glycine–HCl buffer, pH 2.5, and the eluted fractions were adjusted immediately to PH 7.

4.4. Purification of kiwifruit cortex cysteine proteinase inhibitors

Fresh kiwifruit cortex (200 g) was suspended (1/3, v/v) and briefly homogenized in 100 mM sodium citrate, pH 3, containing 2 mM EDTA, 2 mM EGTA, 1% (v/v) Tween 20, 10 mM DTT, 1% (v/v) 2-mercaptoethanol (2-ME), 0.1% (w/v) PEG6000, 0.6 mM PMSF and 10 mg/ml PVPP (Ryan et al., 1998). The extract was filtered through Miracloth and centrifuged at 13,000 *g* for 30 min at 4 °C. Since kiwifruits are rich in cysteine proteinase activity, which is mostly due to actinidain, we inactivated this proteinase by incubation of the supernatant at 60 °C for 10 min. After cooling and centrifugation as above, the supernatant was neutralised, centrifuged again and concentrated using the VivaFlow 200 tangential flow module with a 5000 MWCO PES membrane (Sartorious). The concentrate was applied to a papain affinity column and fractions with papain inhibitory activity were pooled, concentrated using a stirred cell with a 3000 MWCO membrane (Amicon, type YM) and buffer exchanged to 50 mM sodium formate buffer pH 4.0. The sample was then applied to a HiTrap SP, 5 ml column (Amersham Pharmacia) and eluted, at a flow rate of 1 ml/ min, with a 1 M NaCl gradient in 50 mM sodium formate pH 4.0. Fractions of the eluted protein peak were applied to a Vydac C-18 reverse-phase column (25×0.46 cm) (Altech, Deerfield, IL), equilibrated with 10% acetonitrile in 0.1% TFA in water, and eluted (1 ml/ min) with a gradient (10–90%) of acetonitrile in 0.1% TFA in water. Peak fractions containing papain inhibitory activity (tested with the more sensitive substrate Ala-Leu-Lys-AMC) were collected for further investigation.

4.5. Purification of the seed cysteine proteinase inhibitor

Extraction of seeds (60 g) was as described for the cortex KCPI except that the citrate buffer was replaced with100 mM bis-tris–propane, pH 7.0, containing 1 M NaCl, and no heat treatment was applied as cysteine proteinase activity in seeds is negligible compared to that in the cortex. The supernatant was chromatographed on a Superdex G75 column equilibrated with 0.1 M Tris–HCl pH 8.0. Papain inhibitory fractions were pooled, concentrated and purified on an inactive-papain affinity column followed by HiTrap SP and Vydac C-18 columns, as described for the cortex KCPI.

4.6. Purification of actinidain

Actinidain was purified from kiwifruit cortex by covalent chromatography on Thiopropyl-Sepharose (1.5 g gel) as described by Brocklehurst et al. (1981). Purified fractions (possessing a single protein band on silver-stained SDS gel) were used for kinetic studies.

4.7. SDS–PAGE

NuPAGE™ 12% bis–tris gels (Invitrogen) were run in MES-SDS Running Buffer (Invitrogen). SeeBlue Plus2 Pre-stained Standard (Invitrogen) was used for Mr determination and the gels were silver-stained using a standard protocol (Ryan et al., 1998) or using Colloidal Coomassie stain (Neuhoff et al., 1988).

4.8. Proteinase inhibition assays

During the purification procedure, fractions were tested for possible inhibition of pepsin, trypsin, chymotrypsin or papain. The purified CPI was also tested for its ability to inhibit trypsin, chymotrypsin, pepsin and a number of cysteine proteinases. Enzyme and substrate sources and assay buffers are listed in Table 2.

Assays to measure the presence of inhibitor were performed in a 96-well white fluorescent micro-titre plate (A/S Nunc, Roskilde, Denmark) as previously reported (Ryan et al., 1998). An appropriate amount of the proteinase (Table 2) in buffer solution (100 μl) was added to the inhibitor (5–20 μl) and the mixture was incubated at 25 °C for 10–15 min before adding 10 μl of the fluorescent substrate (2.5 mM, in DMF). Rates were calculated from linear regression of the data using in-house generated software (Christeller et al., 1992).

4.9. Quantification of the cysteine proteinase inhibitor and determination of the K_I

The active site concentration of the cysteine proteinase was first determined by titration with E-64 (where applicable) at high concentrations of enzyme (i.e. well above the measured K_I for the CPI) and KCPI concentration was determined by titration against the calibrated papain, again at high concentrations of papain. The K_I was then determined by titration of the inhibitor using calibrated enzyme at concentrations of the enzyme close to the K_I. Where the enzyme was not quantitatively inhibited by E-64, KCPI concentration was pre-determined from papain inhibition, as described above, and then used to calibrate the enzyme assuming interaction at a 1:1 molar ratio.

The quantification of KCPI in crude kiwifruit cortex extract was also done by titration, of actinidain-inactivated and neutralized kiwifruit extract, against papain, at high concentrations of papain.

4.10. Time course of papain inhibition by KCPI1

We measured the rate constants (k_{ass} and k_{diss}) for the interaction of KCPI1 with papain using a Perkin Elmer LS50B spectrofluorimeter under reaction conditions as described for the equilibrium measurements. The rate of proteolysis was initially followed for several minutes before inhibitor was added and the time course following this addition was analysed to extract the rate constants for interaction of the inhibitor with the proteinase (Bieth, 1995; Copeland, 2000).

4.11. Mass spectrometry

The mass of the purified CPI was determined by a MALDI–TOF Finnigan Lasermat 2000 mass spectrometer at the Protein Microchemistry Facility, Department of Biochemistry, University of Otago.

4.12. Protein sequencing

N-terminal amino acids of the CPI were sequenced by the Protein Microchemistry Facility, Department of Biochemistry, University of Otago. Analyses were undertaken using narrow bore HPLC of PTC derivatives (Hubbard, 1995). Where two sequences were observed in the one sample, they were discriminated because they were present in different proportions.

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