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Carboxy terminal extended phytocystatins are bifunctional inhibitors of papain and legumain cysteine proteinases

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Abstract **Plant legumains are cysteine proteinases putatively involved in processing endogenous proteins. Phytocystatins (PhyCys) have been described as plant inhibitors of papain-like cysteine proteinases. Some PhyCys contain a carboxy terminal extension with an amino acid motif (SNSL) similar to that involved in the inhibition of legumain-like proteins by human cystatins. The role of these carboxy terminal extended PhyCys as inhibitors of legumain-like cysteine proteinases is here shown by in vitro inhibition of human legumain and legumain-like activities from barley extracts. Moreover, site-directed mutagenesis has demonstrated that the asparagine of the SNSL motif is essential in this inhibition. We prove for first time the existence of legumain inhibitors in plants.**

Keywords: Barley; Cystatin; Cysteine proteinase inhibitor; Legumain; Papain

1. Introduction

Legumains are a family of Asn-specific cysteine proteinases (family C13; MEROPS peptidase database, http:merops.sanger.ac.uk) that have been described in animals and plants [1,2]. In plants, there is abundant evidence that legumains perform a protein-processing function that causes a limited proteolysis of precursor proteins [3]. In spite of their important role, it has not been reported any legumain-like inhibitor from plants.

Cystatins are proteinaceous inhibitors of papain-like cysteine proteinases (family C1A). Several animal cystatins also inhibit cysteine proteinases of the legumain-related family. Phytocystatins (PhyCys) are specific inhibitors from plants that share with animal cystatins the three motifs involved in the interaction with papain-like enzymes: the reactive site QxVxG, one or two glycine residues in the N-terminal part of the protein and a tryptophan located down-stream of the reactive site. In addition, PhyCys have a consensus sequence ([LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N) that conforms to a predicted secondary α-helix structure and are devoid both, of disulphide bonds and of putative glycosilation sites [4]. Most of the PhyCys are small proteins with a molecular mass ranging from 11 to 16 kDa, although some of them contain a carboxy-terminal extension and have molecular masses of ≈23 kDa [5,6]. The function of this C-tail has not yet been determined.

In the present study, we demonstrate that the carboxy-terminal extensions present in some PhyCys (barley HvCPI-4 and strawberry FaCPI-1) are able to inhibit in vitro both human legumain and the legumain-like activity present in barley protein extracts.

2. Materials and methods

2.1. Cystatin sequence comparisons and bioinformatics analyses

Analysis of DNA and comparisons of deduced protein sequences were done with the current bioinformatics tools. Alignments of amino acid sequences were performed at the DNA Data Bank of Japan (http://www.ddbj.nlg.ac.jp) using the CLUSTALW program [7] with default parameters. The three-dimensional structure of the carboxy terminal amino acid sequence of HvCPI-4 was modelled by the automated SWISS-MODEL program [8,9]. The known crystal structure of the human cystatin C (PDB identifier 1G96) was used to construct the homology-based model. Structure analysis was performed using RasMol 2.7 program [10].

2.2. Expression and purification of recombinant cystatins from *Escherichia coli*

The constructs for barley HvCPI-1–6 [11] and strawberry FaCPI-1 [6] protein expression were introduced in *E. coli* BL21 CodonPlus (Stratagene). Bacterial cells containing the recombinant plasmids were grown at 37 °C to an OD_{550} of *ca.* 0.5 and induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 2 h, harvested and processed. The fusion proteins with a histidine tail were purified using a His-Bind Resin (Novagen) following the manufacturer's instructions, and purification checked by SDS–PAGE.

Two point mutants of the barley cystatin *Icy4* cDNA devoid of the signal peptide sequences were obtained using site-directed mutagenesis by a recombinant PCR approach as described in [12]. P1 and P2 were used as external primers and P3–P6 as overlapping internal primers, which contained the base substitutions (in bold): P1: 5′-gat**gg**taccatcggaaccatggctagc-3′; P2: 5′-tcaaagctttcactggctgctagattc-3′; P3: 5′-gagc**c**gacggtggcc-3′; P4: 5′-ggccaccgtc**gg**ctc-3′; P5: 5′-gagaggtcgaa**g**tcc-3′; P6: 5′-gga**c**ttcgacctctc-3′. The nucleotide changes introduced, which were checked by DNA sequencing, produced the following amino acid alterations in the wild type HvCPI-4 protein: $Q^{86} \rightarrow P$ and $N^{177} \rightarrow K$ (Table 1). In addition, eight truncated variants were generated by PCR using the following primers and DNA templates. The Nterm-ΔA^{142} and Nterm-ΔA^{142} ($Q^{86} \rightarrow P$) variants (P1 and P7: 5′-gacggtaccacctccttcaccatc-3′ primers), and the Nterm-ΔL^{150} and Nterm-ΔL^{150} ($Q^{86} \rightarrow P$) variants (P1 and P8: 5′-gacggtaccggcgccaagagaggg-3′ primers) were made using as DNA template the *Icy4* wild type cDNA and the $Q^{86} \rightarrow P$ point mutant. The ΔT^{143}-Cterm and ΔT^{143}-Cterm ($N^{177} \rightarrow K$) variants (P9: 5′-gacaagctttcaggcgtccccggtgtg-3′ and P2 primers), and the ΔG^{151}-Cterm and

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Abbreviation: PhyCys, phytocystatins

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Table 1
Schematic amino acid sequences of the HvCPI-4 cystatin and its protein variants

Variants	Schematic sequence
HvCPI-4	QTVAG SNSL
HvCPI-4 $Q^{86}\rightarrow P$	PTVAG SNSL
HvCPI-4 $N^{177}\rightarrow K$	QTVAG SKSL
HvCPI-4 Nterm-ΔA^{142}	QTVAG
HvCPI-4 Nterm-ΔA^{142}($Q^{86}\rightarrow P$)	PTVAG
HvCPI-4 Nterm-ΔL^{150}	QTVAG
HvCPI-4 Nterm-ΔL^{150}($Q^{86}\rightarrow P$)	PTVAG
HvCPI-4 ΔT^{143}-Cterm	SNSL
HvCPI-4 ΔT^{143}-Cterm($N^{177}\rightarrow K$)	SKSL
HvCPI-4 ΔG^{151}-Cterm	SNSL
HvCPI-4 ΔG^{151}-Cterm($N^{177}\rightarrow K$)	SKSL

The grey square shows the papain-like inhibitory site. The square indicating the putative legumain-like inhibitory site is scratched.

ΔG^{151}-Cterm ($N^{177} \rightarrow K$) variants (P10: 5′-gacaagcttttagaggtcggagatggtg-3′ and P2 primers) were made using the *Icy4* wild type cDNA and the $N^{177} \rightarrow K$ point mutant as templates. All the resultant barley cystatin variants (Table 1) were digested with KpnI and HindIII (underlined) and inserted in frame into the fusion vector pRSETB (Invitrogen). Then, the recombinant proteins were purified to homogeneity as described above. Their electrophoretic mobilities in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis were consistent with the changes introduced (data not shown).

2.3. Cysteine-proteinase inhibitory activity

Inhibitory activity of the recombinant proteins purified from *E. coli* was tested against commercial papain (Sigma; EC 3.4.22.2) as described in [13] using BANA (*N*-benzoyl-DL-arginine-β-naphthylamide) as substrate. Inhibitory activity against commercial human legumain (R&D Systems; EC 3.4.22.34) was tested using Z-A-A-N-AMC (*N*-carbobenzoxyloxy-Ala-Ala-Asn-7-amido-4-methylcoumarin) as substrate. Briefly, the inhibitor at a concentration of 1 μM was incubated with 25 ng of proteinase for 10 min at room temperature in a buffer containing 50 mM sodium phosphate pH 6.0, 150 mM NaCl, 2 mM EDTA and 1 mM DTT. Then, the substrate at a concentration of 25 μM was added and the reactions incubated for 1 h 30 min at 30 °C. Protein concentrations were quantified by the BioRad kit, with bovine serum albumin (BSA) as standard and the K_i values were determined from Dixon plots (1/V versus [I]).

2.4. Inhibitory activity of cystatins against barley protein extracts

For inhibitory proteinase assays, seeds of barley (*Hordeum vulgare*) cv. Bomi were germinated at 22 °C in the dark for 7 days, and used to collect samples of roots. Developing endosperms of 18 days after flowering were prepared from kernels of plants grown in a greenhouse at 18 °C under an 18/6 day/night photoperiod. All samples were frozen into liquid N_2 and stored at −70 °C until used for protein extraction.

Samples were ground with mortar and pestle and resuspended in 0.15 M NaCl, 50 mM sodium phosphate pH 6.0, 2 mM EDTA for 1 h at 4 °C. After centrifugation for 15 min at 10000 rpm, supernatants were recovered and their protein content quantified. Inhibitory activity of recombinant cystatins was tested in vitro against protein extracts using Z-A-A-N-AMC as substrate, as described above. Different concentrations of the inhibitor (0 nM, 25 nM, 50 nM, 100 nM, 200 nM, and 500 nM) were incubated with 10 μg of a soluble endosperm protein extract or 5 μg of a soluble root protein extract, at 25 °C for 10 min prior to addition of substrate. Results were expressed as a percentage of proteinase activity relative to that in the absence of the inhibitor, and the inhibitory cystatin doses for 50% proteinase activity inhibition (ID_{50}) for each cystatin was calculated. All assays were carried out in triplicate.

3. Results

3.1. Comparison of the C-terminal tails from carboxy terminal extended PhyCys

In order to evaluate the similarities in the C-terminal tails of PhyCys with a carboxy terminal domain, an alignment of the amino acid sequences of fifteen carboxy terminal extensions from PhyCys found in the databanks was performed (Fig. 1). A survey of the alignment indicates strong amino acid conservation in the compared sequences that suggest a conserved role in the plant. Interestingly, the amino acid motif SNSL was detected in all sequences, and this motif resembles the sequence involved in the inhibition of legumains by human cystatins.

To test the amino acid similarities between the C-terminal tails and the human cystatins, an alignment between the amino acid sequence of the carboxy terminal tail of HvCPI-4 and the amino acid mature sequence of the strongest human legumain inhibitor, the cystatin E/M, was done (Fig. 2). Strong amino acid conservation was observed at the N-terminal region of the molecules, where the domain involved in legumain inhibition by human cystatins is located. The three-dimensional structure of the C-terminal tail of HvCPI-4 was modelled using as template the known dimeric structure of cystatin C [14], which is also able to inhibit legumain. Only the N-terminal part of HvCPI-4 was predicted. A similar structure containing the α-helix was observed in the region surrounding the backside loop (BSL), where the asparagine responsible of legumain inhibition by human cystatins is located (Fig. 2). All these results suggested that the C-terminal tails of the carboxy terminal extended PhyCys could be plant inhibitors for legumain proteinases.

3.2. Differential cysteine proteinase inhibition of the phytocystatins

To prove the role of the carboxy terminal extensions of PhyCys as legumain inhibitors, the inhibitory properties of two C-terminal extended PhyCys, HvCPI-4 and FaCPI-1 and five not C-terminal extended barley PhyCys with papain inhibitory

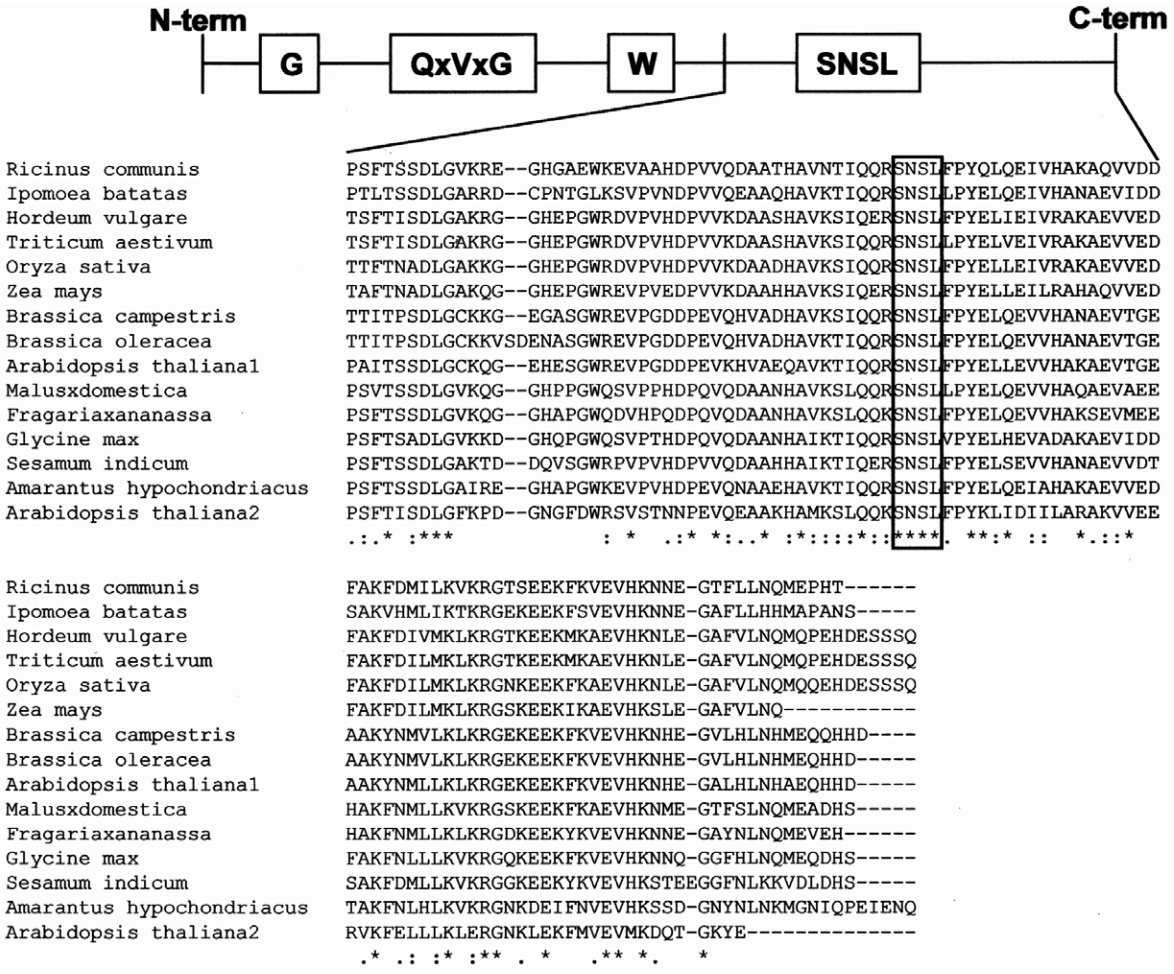

Fig. 1. Alignment of the C-terminal domain present in 15 PhyCys. A schematic representation of an entire cystatin showing the putative functional motifs is attached. The conserved amino acids are indicated by an asterisk and strong and weak conservative changes are marked with colons and dots, respectively. A square marks the conserved SNSL motif. GenBank Accession Numbers corresponding to the sequences are as follows: *Amarantus hypochondriacus* AhCPI (DQ792503); *Arabidopsis thaliana1* AtCYS-6 (NM180239); *Arabidopsis thaliana2* AtCYS-7 (NM120593); *Brassica campestris* BCPI-1(L41355); *Brassica oleracea* BoCPI-1 (AY065838); *Fragariaxananassa* FaCPI-1 (AJ845186); *Glycine max* (D31700); *Hordeum vulgare* HvCPI-4 (AJ748344); *Ipomoea batatas* CYSPI (AF241536); *Malusxdomestica* (AY173139); *Oryza sativa* OC-XII (AK119511); *Ricinus communis* (Z49697); *Sesamum indicum* (AF240007); *Triticum aestivum* TaMDC1 (AB223039); *Zea mays* ZmCC4 (AM055633).

activity, HvCPI-1, 2, 3, 5 and 6 [11], were tested. Whereas all PhyCys were able to inhibit papain, only the two C-terminal extended PhyCys competitively inhibited in vitro the proteinase activity of human legumain (Table 2) using Z-A-A-N-AMC as substrate. To test if the carboxy terminal region, and specifically, if the SNSL motif was involved in this inhibition, several variants of the HvCPI-4 barley cystatin were constructed (Table 1). The variants were expressed in *E. coli* as fusion proteins, purified and assayed against papain and legumain (Table 2). The K_i values against both enzymes were determined. The variants that conserved the first part of the protein, in which the structure that supports a papain-like inhibitory activity is located, were able to inhibit papain with the exception of the variants in which the glutamine of the reactive center (QTVAG) was changed to a proline residue. Likewise, all the variants that share the second part of the protein, in which the putative legumain-like inhibitory motif (SNSL) is located, presented inhibitory activity against human legumain. When the asparagine of this motif was changed to a lysine, the variant lacked its legumain-like inhibitory properties. Similar legumain inhibition was found when the HvCPI-4 ΔG^{151}-Cterm variant, a truncated construction that lacks the eight first amino acids of the C-tail, was tested.

To study if PhyCys were able to inhibit the endogenous barley legumains, in vitro assays against the legumain activity present in barley extracts were performed with the barley cystatins, the strawberry cystatin, and all the HvCPI-4 variants. The assays were carried out by adding increasing concentrations of each cystatin to the soluble extract proteins, and the inhibitory activity was measured by the inhibition of legumain-like proteinase activity. As expected, the cystatins able to inhibit human legumain also inhibit the barley legumain-like proteinase activities in both developing endosperms and roots. The inhibitory doses for 50% proteinase activity inhibition were higher for root legumain activity than for endosperm one. The endogenous barley legumain-like activities were inhibited by iodoacetamide, and not inhibited by the non-legumain inhibitors leupeptin and E64, which corroborates that these activities were performed by legumain-like enzymes (data not shown). As expected, no proteinase inhibition was ob-

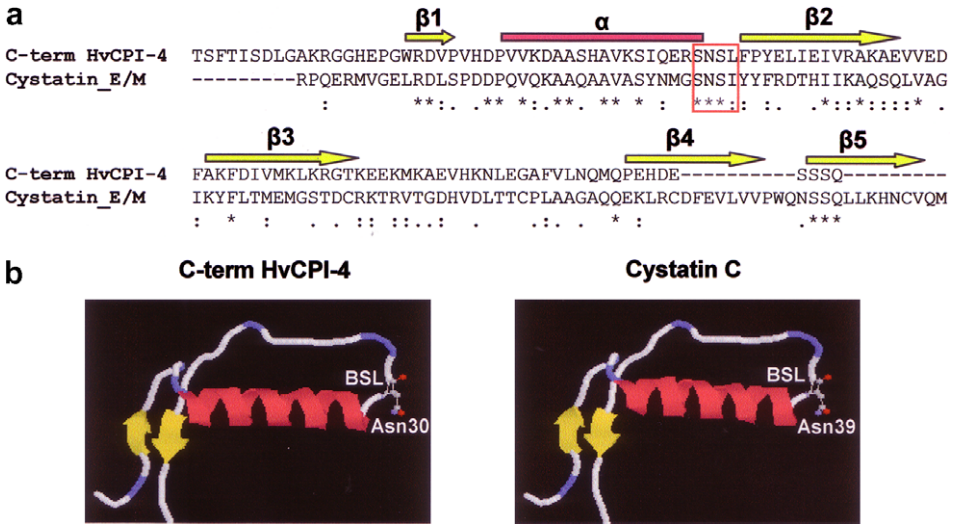

Fig. 2. (a) Amino acid alignment of the C-terminal domain of the barley HvCPI-4 cystatin and the mature human E/M cystatin (GenBank Number NM001323). The putative location of the secondary structures (α-helix and β-sheets) is included. The conserved amino acids are indicated by an asterisk and strong and weak conservative changes are marked by colons and dots, respectively. A square marks the conserved SNSL motif. (b) Ribbon plot of the region surrounding the backside loop of the human cystatin C and the predicted carboxy terminal extension of the barley cystatin HvCPI-4. The three-dimensional structure of barley cystatin was predicted using the automated SWISS-MODEL program with the human cystatin C as a template. Figure prepared with RasMol 2.7. The backside loop (BSL) and the asparagine present in the legumain-like inhibitory motif are indicated.

Table 2
Inhibition constant (K_i) values of the six barley cystatins (HvCPI-1–6), the HvCPI-4 variants, and the strawberry FaCPI-1 protein against papain and legumain; and inhibition of the legumain-like activities in protein extracts from barley tissues

Cystatins	K_i (M)		ID_{50} (μM)	
	Papain	Legumain	Developing endosperm	Root
HvCPI-1	2.0×10^{-8}	ni[a]	ni	ni
HvCPI-2	5.1×10^{-8}	ni	ni	ni
HvCPI-3	4.0×10^{-8}	ni	ni	ni
HvCPI-4	2.2×10^{-7}	1.9×10^{-7}	38	120
HvCPI-5	6.3×10^{-8}	ni	ni	ni
HvCPI-6	1.7×10^{-9}	ni	ni	ni
HvCPI-4 $Q^{86} \rightarrow P$	ni	1.3×10^{-7}	33	110
HvCPI-4 $N^{177} \rightarrow K$	2.6×10^{-8}	ni	ni	ni
HvCPI-4 Nterm-ΔA^{142}	5.6×10^{-8}	ni	ni	ni
HvCPI-4 Nterm-ΔA^{142}($Q^{86} \rightarrow P$)	ni	ni	ni	ni
HvCPI-4 Nterm-ΔL^{150}	1.7×10^{-7}	ni	ni	ni
HvCPI-4 Nterm-ΔL^{150}($Q^{86} \rightarrow P$)	ni	ni	ni	ni
HvCPI-4 ΔT^{143}-Cterm	ni	1.9×10^{-7}	18	68
HvCPI-4 ΔT^{143}-Cterm($N^{177} \rightarrow K$)	ni	ni	ni	ni
HvCPI-4 ΔG^{151}-Cterm	ni	2.9×10^{-7}	22	91
HvCPI-4 ΔG^{151}-Cterm($N^{177} \rightarrow K$)	ni	ni	ni	ni
FaCPI-1	1.9×10^{-9}	7.6×10^{-7}	20	200

[a]ni indicated no inhibitory activity observed at a 5 μM concentration of the inhibitor.

served with the protein purified from *E. coli* transformed with the expression vector without insert used as a negative control (data not shown).

4. Discussion

Legumain is the name that Kembhavi et al. [15] gave to an endopeptidase present in the moth bean that is selective for the cleavage of asparaginyl bonds. From this study, a common inhibition by iodoacetamide and the lack of inhibition by E64 and leupeptin for legumain-like proteins stated that they are a novel group of cysteine proteinases apart of the papain-like family. Cystatins are potent inhibitors of the papain-like family of cysteine proteinases. However, inhibitory assays using chicken egg cystatin demonstrated that both plant and animal legumain-like proteins are susceptible to cystatin inhibition [1,15,16]. Since the protein fold of the legumain family members seems to be much more closely related to the caspases and gingipain than to papain [17], Alvarez-Fernandez et al. [18] suggested that the inhibitory sites against papain and legumain-like cysteine proteinases are far located in the tridimensional structure of cystatins. Besides, in a comparative study of the legumain inhibitory properties of eight human proteins belonging to the cystatin superfamily, they found that only three of the type 2 cystatins (cystatins C, E/M and F) were able to inhibit pig legumain [18]. These proteins share a NS or ND motif located in the loop between the α-helix and the second β-strand. The presence of a lysine between the asparagine and the aspartic acid probably impedes legumain inhibition by cystatin D [19].

In plants, legumains or VPE (vacuolar processing enzymes) catalyze an Asn- and Asp-specific limited proteolysis. They have been involved in several processes. During germination, legumains act as processing enzymes, and contribute to the activation of papain-like cysteine proteinases to degrade storage proteins [20–22]. A role in defense against pathogens executing programmed cell death due to the caspase activity observed for several legumains have also been proposed [23,24]. In spite of these important functions, a plant proteinaceous legumain inhibitor had not been described. The analysis of the amino acid sequences of the carboxy terminal extended PhyCys revealed the presence of a common motif SNSL in a predicted three-dimensional structure similar to the backside loop of cystatins. This motif shared the NS amino acids located in the legumain inhibitory backside loop of the human E/M cystatin, and was not found in the backside loop of the papain-like inhibitory domain of barley cystatins. The inhibitory capacity against human legumain of the carboxy terminal extension of several PhyCys was confirmed, and the importance of the Asn residue of the SNSL motif determined by its change to a Lys residue. Likewise, the inhibition of the legumain-like activity observed in protein extracts from barley roots and developing endosperms confirms that the carboxy terminal extensions of these cystatins may act in vivo as endogenous regulators of the legumain activity.

In conclusion, we have determined the first proteinaceous legumain-like proteinase inhibitor from plants. Interestingly, the comparison of the inhibitory features between animal and plant legumain-like cystatin inhibitors shows a different evolution. In animals, the papain and legumain-like inhibitory motifs are present in the same amino acid cystatin domain. In plants, a second domain, probably derived from a duplication of an ancestral cystatin, retained the legumain-like inhibitory properties, which were lacked by the papain-like inhibitory domain.

In addition, the fact that carboxy terminal extended PhyCys act as bifunctional proteinase inhibitors simultaneously inhibiting two kinds of proteases makes them good candidates to be used as transgenes against agronomically important pests and pathogens.

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