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Purification and Characterization of Two Cysteine Proteinases from Potato Leaves and the Mode of Their Inhibition with Endogenous Inhibitors

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Two cysteine proteinases, PLCP-1 and PLCP-2, were purified from potato leaves (*Solanum tuberosum* L.). SDS-PAGE of PLCP-2 gave a single band with M_r of 23400 and PLCP-1 gave a doublet within the same M_r range. Isoelectric focusing of PLCP-2 revealed two bands with pI = 4.6 and 4.9. Both enzymes demonstrate pH optima and maximum stability at slightly acidic pH, and strong inhibition by L-*trans*-epoxysuccionylleucylamido(4-guanidino)butane (E-64), cystatin C and stefin A, enabling them to be assigned to the papain family of cysteine proteinases. PLCP-1 and PLCP-2 were inhibited by Kunitz-type cysteine proteinase inhibitors (PCPIs) and multicystatin, all isolated from potato tubers. Among PCPIs, the strongest inhibitors were PCPI 9.4, with K_i in the 10^{-8} M range, and PCPI 8.3 in the 10^{-7} M range, while K_is for PCPI 6.6 and PCPI 5.4 were in the 10^{-6} M range. Multicystatin was the most potent inhibitor of both proteinases with K_i of about 0.5 nmol dm^{-3}. The stoichiometry of inhibition of both proteinases with multicystatin was 1:4 (inhibitor : proteinase). The possible physiological significance of these endogenous inhibitors, also present in potato leaves, is discussed. PLCP-1 and PLCP-2 could not be differentiated in terms of their K_is.

Keywords
cysteine proteinase
leaf
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Solanum tuberosum L.
potato Kunitz peptidase inhibitor-C
multicystatin

INTRODUCTION

Proteolytic enzymes play a crucial role in plant physiology, development and environmental response. They are involved in controlling the metabolism, nutrient mobilization, maturation of zymogens and peptide hormones by limited proteolysis, modulating levels of receptors and in selective proteolysis mediated by the ubiquitin/26S proteasome pathway.[1,2] In plant cells, there is a great variety of proteinases of all classes, which have been characterized to different extents. Cysteine proteinases, mostly belonging to the papain (C1) and legumain (C13) families, are present in almost all plant species and in most plant tissue.[3–5] As reviewed by Beers *et al.*,[6] papain-like proteinases are involved in protein remobilization during seed germination and organ senescence, and are implicated in numerous programmed cell death processes, plant defense mechanisms and disease resistance. In potato plants, mostly tubers have been investigated for cysteine proteinases. Kitamura and Maruyama isolated and characterized a 28 kDa cysteine proteinase from sprouting potato tubers, which was shown to be associated with a decrease in total soluble protein content.[7,8] A similar enzyme was detected in dormant potato tubers.[9] Michaud *et al.*, when monitoring general endoproteolytic activity during potato sprouting, detected an increase of cysteine protein-

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ase activity due to gradual appearance of at least six new cysteine proteinase forms, while only one cysteine proteinase form was present during early sprouting.[10] Kumar *et al.* detected highly active cysteine proteinases of 75, 90 and 100 kDa, which appeared in long-term ageing potato tubers.[11] One of the few reports dealing with potato leaves described the immunolocalization of a cysteine proteinase named PLCP-2 in protein bodies, in vacuoles and in the cell wall. Proteinase was detected also in shoots, tips, stems and roots.[12] In potato leaves, the expression of a cysteine proteinase cDNA was observed at an early stage of resistance acquisition following fungal attack.[13] Recently, in the related plant – tomato, a secreted papain-like proteinase Rcr3 was recognized to be required for the function of the disease resistance gene *Cf-2*.[14]

Several systems of protein degradation operate in plant cells, each requiring different mechanisms or modes of control. Among them, regulation of protease activity by endogenous inhibitors in appropriate locations may be most effective.[3,15] In potato tubers, two different types of cysteine proteinase inhibitor (CPI) are present. The first type potato cysteine peptidase inhibitors (PCPIs) with M_r of 22–25 kDa and differing in isoelectric point belong, on the basis of their primary structures, to the Kunitz-type soybean trypsin inhibitor superfamily with further classification to the PKPI-C homology group of the PKPI family.[16–21] Although they are potent inhibitors of the lysosomal cysteine proteinases cathepsin L and papain, they differ considerably in their inhibitory activity, and at least one of them possesses a weak inhibitory activity against trypsin.[16,19,22,23] The second type of potato cysteine proteinase inhibitor, with M_r of 85 kDa, isolated from tubers is named multicystatin, since it is composed of 8 tandem cystatin domains capable of binding 8 papain molecules at the same time.[24] Both CPI types have been detected in potato leaves as well.[24,25]

Cysteine proteinases and their inhibitors in mature leaves growing under steady state turnover and their mutual relations have not been studied thoroughly. The presence of both at the same or similar locations in leaves raises the question of the strength and specificity of their potential interactions. In the present work, we present the isolation and characterization of two cysteine proteinases, PLCP-1 and PLCP-2, from mature potato leaves and consider their interaction with endogenous inhibitors PCPIs and multicystatin in order to obtain an insight into their possible physiological regulation.

EXPERIMENTAL

Plant Material

Potato plants (*Solanum tuberosum* L. cv. Desirée) were grown in the field. At the stage preceding flowering, mature leaves were harvested and stored at –20 °C.

Purification of Cysteine Proteinases

Protein extract from 1 kg of potato leaves was obtained by a modified method of Denison[26] as described earlier.[26,27] It was concentrated in an ultrafiltration cell (Amicon Corp., USA), using a YM-10 membrane and applied to a Sephacryl S-200 (Pharmacia-LKB) column (4 × 110 cm) equilibrated with 0.1 M phosphate buffer (pH = 6.0), containing 0.3 mol dm^{-3} NaCl and 1 mmol dm^{-3} EDTA. Fractions active against Z-Phe-Arg-7-(4-methyl)coumarylamide (Z-Phe-Arg-MCA) were collected, concentrated, dialyzed against 0.02 mol dm^{-3} sodium acetate buffer (pH = 5.2), containing 1 mmol dm^{-3} EDTA, and applied to a CM Sephadex C-50 (Pharmacia-LKB) column (2.8 × 30 cm). One part of the active material, eluted in the break-through peak, was dialyzed against 0.05 mmol dm^{-3} phosphate buffer (pH = 6.9), containing 1 mmol dm^{-3} EDTA, and applied to a DEAE Sephacel (Pharmacia-LKB) column (2.7 x 37 cm). Active fractions eluted from this column after application of NaCl gradient (0–0.75 mol dm^{-3}) were concentrated, reduced with 2 mmol dm^{-3} dithiothreitol (DTT) for 30 min at room temperature and separated from low molecular thiols on a Sephadex G-25 (Pharmacia-LKB) column (2 × 20 cm) equilibrated with sodium acetate buffer (pH = 4.5), containing 0.3 mol dm^{-3} NaCl and 1 mmol dm^{-3} EDTA. The eluted proteins were mixed overnight with 60 g (wet weight) of Thiopropyl Sepharose 6B (Pharmacia-LKB), equilibrated with the same buffer. Batch-wise washing of unbound material with pH = 4.5 buffer and with 0.1 M phosphate buffer (pH = 6.5), containing 0.3 mol dm^{-3} NaCl and 1 mmol dm^{-3} EDTA, was followed by the elution of the bound proteins with 20 mmol dm^{-3} cysteine in the latter buffer.

The part of the active material that was eluted from the CM Sephadex C-50 column after the NaCl gradient application was further purified on Thiopropyl Sepharose as described above.

Protein Determination

Protein concentration in unpurified samples was determined according to Bradford[28] using the Bio-Rad Protein Assay Kit, and bovine serum albumin as a standard. Protein concentrations in eluted fractions and in purified samples were determined by measuring absorbance at 280 nm. A_{1cm} at 280 nm for isolated cysteine proteinases (γ = 10 g/L) was assumed to be 20, the average value for three plant cysteine proteinases.[29] Concentrations of inhibitors were determined photometrically, taking $A_{280,1cm}$ (10 g/L) to be 11 for PCPIs and 12 for multicystatin.[18,24]

SDS-PAGE and Isoelectric Focusing

Electrophoresis in the presence of 50 g/L SDS was carried out in T = 8–25 % polyacrylamide gradient gels in Tris-acetate buffer (pH = 6.4) on a Pharmacia PhastSystem apparatus (Pharmacia-LKB), as recommended by the manufacturer ($T/\% = \frac{m(\text{polyacrylamide})}{v(\text{gel})} \cdot 100$). The gel was calibrated

with the Pharmacia Low Mass calibration kit and stained with 1 g/L Coomassie brilliant blue R-250.

The same apparatus was used for analytical isoelectric focusing on a commercial precast pH = 3–9 gradient gel, following the instructions provided. A mixture of standard proteins with pI range 3.65–9.3 (Pharmacia broad-pI calibration kit) was run in parallel with the samples. Gels were stained as for SDS-PAGE.

Enzyme Assays and Active Site Titration

During the isolation procedure, cysteine proteinase activities were followed fluorimetrically using Z-Phe-Arg-MCA as a substrate in 10 μmol dm^{-3} final concentration.[30] Assay buffer was 0.1 M phosphate buffer (pH = 6.0), containing 10 mmol dm^{-3} cysteine and 1.5 mmol dm^{-3} EDTA. The activity of aspartic proteinases was followed using fluorescein thiocarbamoyl-hemoglobin (FTC-hemoglobin) as substrate in 0.1 M acetate buffer (pH = 3.3). Soluble peptide products were determined fluorimetrically at 490 nm excitation and 525 nm emission wavelengths, as described for FTC-casein.[31]

Active concentrations of isolated cysteine proteinases were determined by titration with 0.5 μmol dm^{-3} L-*trans*-epoxysuccinylleucylamido(4-guanidino)butane (E-64) (Peptide Research Foundation, Osaka, Japan) using Z-Phe-Arg-*p*-nitroanilide (Z-Phe-Arg-pNA) as substrate at final concentration of 150 μmol dm^{-3}.[30] The product was monitored by spectrophotometry at 405 nm.

Influence of Different Inhibitors on the Activity of Proteinases

Influence of different inhibitors on the activity of cysteine proteinases was tested by incubating each inhibitor at the indicated concentration in the assay buffer with the enzyme for 10 min prior to the addition of Z-Phe-Arg-MCA as a substrate. Iodoacetic acid, iodoacetamide and soybean trypsin inhibitor (SBTI) were from Sigma, Germany, phenylmethylsulfonyl fluoride (PMSF) and pepstatin were from Boehringer Mannheim, Germany. Stefin A and cystatin C were isolated in our laboratory.[32,33]

Inhibition of FTC-hemoglobin activity at pH = 3.3 with pepstatin was assayed at final concentration of the inhibitor of 100 μmol dm^{-3}.

pH Optimum and pH Stability

Dependence of the enzyme activity on pH was determined using a series of buffers prepared from 50 mmol dm^{-3} acetic acid, 50 mmol dm^{-3} 2-(*N*-morpholino)ethanesulfonic acid (MES) and 100 mmol dm^{-3} Tris. The same buffers were used for pH stability determination. 10 μL of the enzyme was incubated in sample buffer for 1 hour at 37 °C. 10 μL aliquots were transferred to 0.5 mL or 0.3 mL assay buffers and tested for activity towards Z-Phe-Arg-MCA or FTC-haemoglobin.

Isolation of Cysteine Proteinase Inhibitors PCPIs and Multicystatin

PCPIs with isoelectric points 5.4, 6.6, 8.3 and 9.4 were isolated from potato tubers of Desirée variety as previously described.[16] Multicystatin was isolated as described by Walsh and Strickland.[24]

Determination of Inhibition Constants K_i *for PCPIs and Multicystatin*

Each enzyme was incubated at an active concentration of about 3 nmol dm^{-3} with different amounts of each inhibitor to obtain non-linear dose response curves of residual activity. The enzyme and inhibitor were preincubated for 15 min at room temperature in 0.1 M phosphate buffer (pH = 6.0), containing 10 mmol dm^{-3} cysteine and 1.5 mmol dm^{-3} EDTA. 10 μL of Z-Phe-Arg-MCA was then added to final concentrations of 10 μmol dm^{-3}, 5 μmol dm^{-3} or 2.5 μmol dm^{-3}. After 10 min of incubation at 37 °C, the reaction was stopped by adding 5 mmol dm^{-3} iodoacetic acid. The released 7-MCA was measured fluorimetrically on a Perkin-Elmer LS 30 fluorimeter. The inhibitor concentrations in the reaction mixtures ranged from 0.08 to 2.4 for PCPIs and 0.02 to 0.06 μmol dm^{-3} for multicystatin. The apparent inhibition constants were obtained graphically using the linear equation derived by Henderson.[34] The influence of the substrate on the true inhibition constant (K_i) was eliminated by extrapolation of $K_{i,app}$ to zero substrate concentration, according to the equation:

$$K_i = K_{i,app}/(1 + [S_0]/K_M),$$

where K_M is Michaelis constant and $[S_0]$ is substrate concentration.

RESULTS

Purification of Cysteine Proteinases and Detection of Aspartic Proteinase in Potato Leaves

The method outlined in Figure 1 is based on three phase partitioning using *t*-butanol and ammonium sulphate.[26,27] Purification was continued by gel filtration and ion exchange chromatography on CM-Sephadex C-50 where activity towards Z-Phe-Arg-MCA was separated into two fractions, one being unbound, containing PLCP-1, and the other eluted after NaCl gradient application, containing PLCP-2 (Figure 2). Unbound material was chromatographed on another ion exchange column (DEAE Sephacel, Figure 3) where Z-Phe-Arg-MCA activity was eluted with a NaCl gradient at the concentration of 0.6–0.7 mol dm^{-3}. From the same column, at 0.1–0.2 mol dm^{-3} NaCl, a material with activity against FTC-haemoglobin at pH = 3.3, was eluted. Both fractions active against Z-Phe-Arg-MCA, eluted with gradients from CM-Sephadex C-50 and from DEAE Sephacel, were separately further purified by covalent chromatography on Thiopropyl

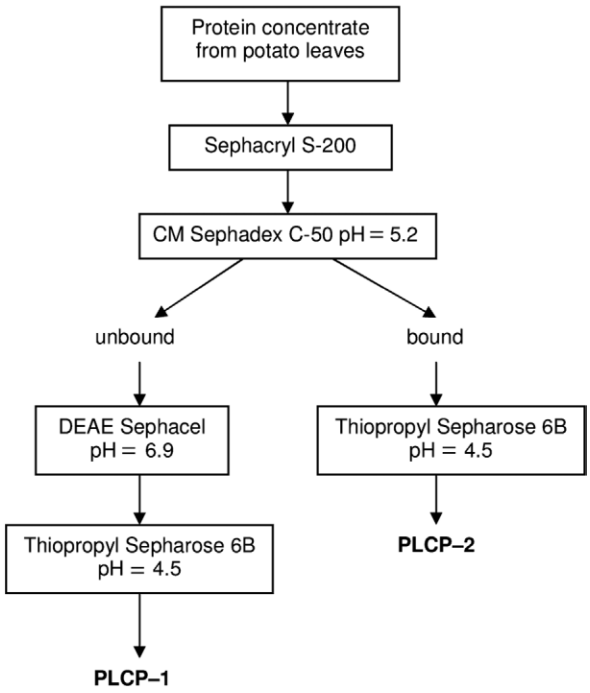

Figure 1. Schematic presentation of the isolation procedure for two cysteine proteinases from potato leaves.

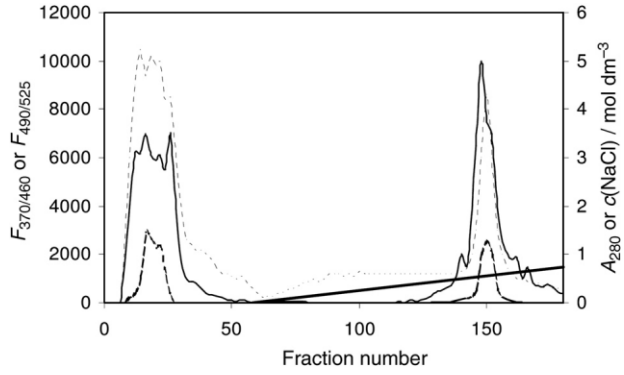

Figure 2. Cation-exchange chromatography on CM Sephadex C-50 at pH = 5.2 of potato leaf concentrate after gel filtration. The solid and dashed lines indicate activities towards Z-Phe-Arg-MCA at pH = 6.0 and FTC-hemoglobin at pH = 3.3, respectively, expressed as $F_{370/460}$ or $F_{490/525}$. The light dashed line indicates A_{280} and the thick line the NaCl gradient.

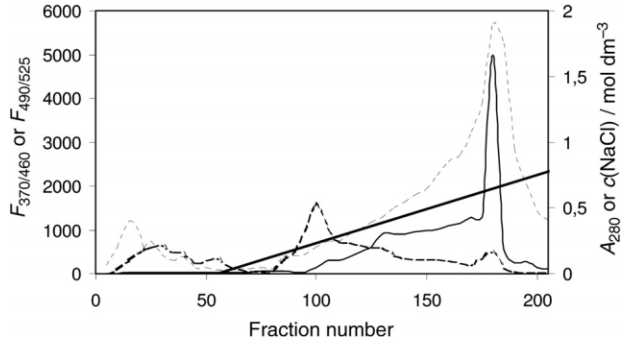

Figure 3. Anion-exchange chromatography of material containing PLCP-1 on a DEAE Sephacel column. The solid and dashed lines indicate activity towards Z-Phe-Arg-MCA at pH = 6.0 and FTC-hemoglobin at pH = 3.3, respectively, expressed as $F_{370/460}$ or $F_{490/525}$. The light dashed line indicates A_{280} and the thick line the NaCl gradient.

Sepharose at pH = 4.5. Quantification of the purification of two cysteine proteinases from 1 kg of potato leaves is presented in Table I. The yields of the two cysteine proteinases were 0.23 mg of PLCP-1 and 0.73 mg of PLCP-2.

The enzyme from DEAE Sephacel fractions active against FTC-hemoglobin was partially characterized by its pH optimum at 3.6 and its 98 % inhibition by pepstatin.

Characterization of Cysteine Proteinases

PLCP-1 and PLCP-2 were analyzed by SDS-PAGE in the presence and absence of reducing agent, where a single band at M_r of 23 400 appeared for PLCP-2 and a doublet, at apparently the same molecular mass, for PLCP-1 (Figure 4). Isoelectric focusing revealed two bands with pI = 4.6 and 4.9 for PLCP-2.

TABLE I. Purification of cysteine proteinases from 1 kg of potato leaves

Purification step	*m* (Protein)	Total activity[(a)]	Specific activity	Purification	Yield
	mg	nmol min^{-1}	nmol min^{-1} mg^{-1}	(-fold)	%
Protein extract	3161	41666	13	1	100
Sephacryl S-200	217	14554	67	5	35
CM-Seph. unbound	75	3908	52	4	7
DEAE Sephacel	12	949	79	6	2
Thiopropyl Seph.					
PLCP-1	0.23	125	545	42	0.3
CM-Seph. bound	40	3958	99	8	7
Thiopropyl Seph.					
PLCP-2	0.73	330	452	34	0.8

(a) Activities were determined against Z-Phe-Arg-MCA.

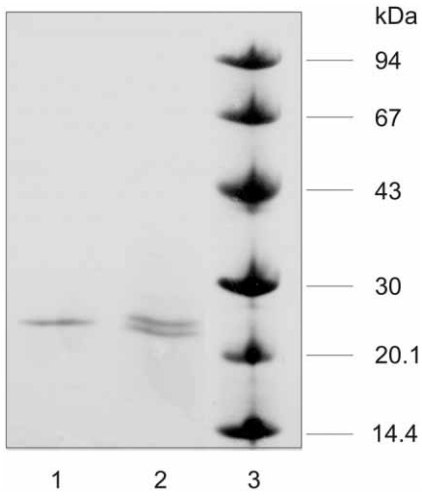

Figure 4. SDS-PAGE of cysteine proteinases from potato leaves in the presence of reducing agent. Lane 1, PLCP-2; lane 2, PLCP-1; lane 3, standard proteins.

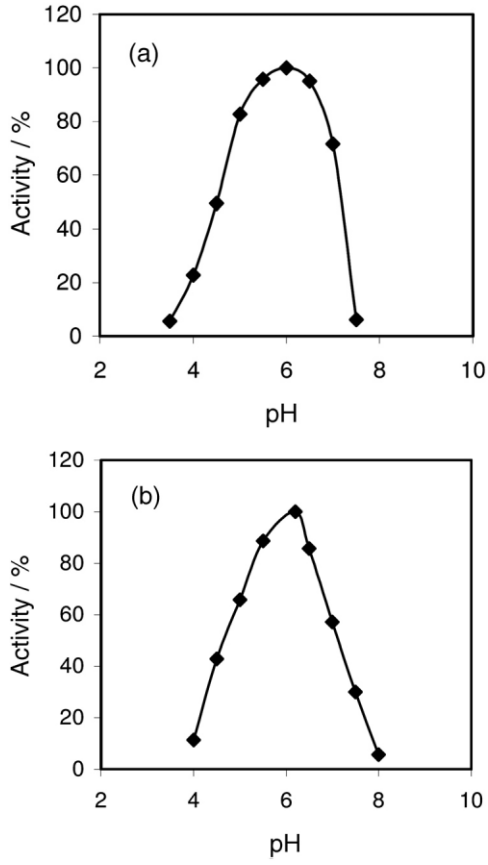

Figure 5. Effect of pH on the activity (a) and stability (b) of PLCP-2. pH optimum and stability were assayed as described in Experimental.

The activities of PLCP-2 was determined as a function of pH in acetic acid/MES/Tris buffers of constant ionic strength using Z-Phe-Arg-MCA as substrate.[35] A bell-shaped curve was obtained with a maximum at pH = 6.0 (Figure 5a). PLCP-1 showed similar pH dependence of activity.

TABLE II. Influence of various protease inhibitors on the activity of cysteine proteinases from potato leaves[(a)]

Inhibitor	Concentration	Activity / %	
	μmol dm^{-3}	PLCP-1	PLCP-2
None		100	100
E-64	2	0	0
Iodoacetic acid	20	10	12
Iodoacetamide	20	18	21
Cystatin C	2	1	
Stefin A	2	0	0
PMSF	100	103	100
SBTI	20	96	99
Pepstatin	100	100	98
$CaCl_2$	1000	97	105
$MgCl_2$	1000	99	98

[(a)] Enzymes were preincubated with inhibitor at the indicated concentration for 10 min in the assay buffer. The reaction started after the addition of Z-Phe-Arg-MCA as a substrate. Activities were determined as described in Experimental.

The stability of PLCP-2 at different pH values is presented in Figure 5b. After 1 hour of incubation at 37 °C, the enzyme retained 50 % of its activity at pH = 4.3 and pH = 7.1, and exhibited the highest stability at pH = 6.2. PLCP-1 showed a similar pattern of pH stability.

The influence of different substances on the activity of PLCP-1 and PLCP-2 is summarized in Table II. Inhibitors of serine (PMSF, SBTI), aspartic (pepstatin) and metallo-proteinases ($CaCl_2$, $MgCl_2$ and EDTA present in all buffers) had practically no influence on the activity of the two proteinases. All the typical inhibitors of cysteine proteinases (E-64, iodoacetic acid, iodoacetamide, cystatin C, stefin A) strongly inhibit both proteinases.

Inhibition of Cysteine Proteinases by Protein Inhibitors from Potato

The K_i values for the inhibition of cysteine proteinases PLCP-1 and PLCP-2 by potato CPIs of Kunitz family (PCPI 5.4, PCPI 6.6, PCPI 8.3 and PCPI 9.4) and multicystatin are given in Table III. For comparison, K_i values for papain, the usual test enzyme, are included. For calculations of K_i, concentrations of PCPIs and multicystatin were based on protein determination. The apparent inhibitor constants correlated linearly with substrate concentration. From this relation, K_M values for hydrolysis of Z-Phe-Arg-MCA by PLCL-1 and PLCP-2 were estimated to be 15 μmol dm^{-3} and 10 μmol dm^{-3}, respectively.

Active concentrations of PLPC-1 and PLCP-2 determined by titration with E-64 were used for titration with multicystatin. A stoichiometric ratio of 1 : 4 (inhibitor : proteinase) was determined for its reaction with both PLCP-1 and PLCP-2 (Figure 6).

TABLE III. Inhibition constants K_i for inhibition of cysteine proteinases from potato leaves with potato cysteine proteinase inhibitors of Kunitz type (PCPIs) and multicystatin[a]

Inhibitor	K_i / μmol dm^{-3}		
	PLCP-1	PLCP-2	Papain
PCPI 5.4	7.5	4.8	n.d.
PCPI 6.6	2.3	0.97	0.05 [b]
PCPI 8.3	0.4	0.32	3.3×10^{-3} [c]
PCPI 9.4	0.077	0.057	0.1 [b]
Multicystatin	0.6×10^{-3}	0.4×10^{-3}	0.1×10^{-3} [d]

[a] Values for papain are from the literature; [b] Ref. 16, [c] Ref. 22, [d] Ref. 24.

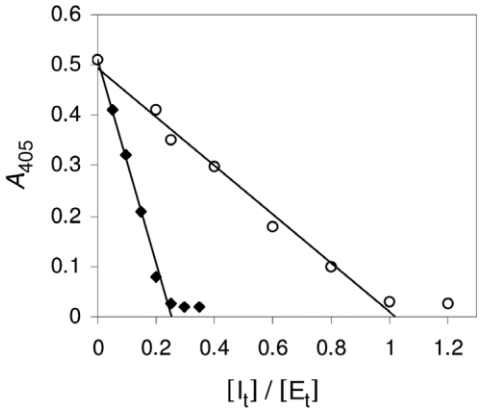

Figure 6. Active-site titration of PLCP-2 and stoichiometric titration of multicystatin with PLCP-2. Proteinase was titrated with 0.5 μmol dm^{-3} E-64 (open circles) to determine active concentration. The same amount of proteinase was titrated with 0.05 μmol dm^{-3} multicystatin (black squares). Residual activities were determined with Z-Phe-Arg-pNA as substrate. $[I_t]$, total inhibitor concentration; $[E_t]$, total enzyme concentration.

DISCUSSION

At the first stages of purification of cysteine proteinases from potato leaves, screening for a range of proteases was performed, which revealed proteolytic activity against FTC-hemoglobin at acidic pH. This probably represents an aspartic proteinase, since the activity was inhibited by pepstatin. In a closely related plant, tomato, an aspartic proteinase was already detected in leaves,[36] and a cDNA from tomato leaf encoding for wound induced aspartic proteinase was also reported.[37] In addition, an aspartic proteinase was isolated from cultured tobacco cells, although this proteinase was only slightly sensitive to inhibition by pepstatin.[38]

The three phase partitioning using t-butanol and ammonium sulphate[26] appears well adapted to preparing a protein concentrate from potato leaves. Many unwanted molecular interactions in the homogenate, *e.g.*, proteolysis or proteinase/inhibitor binding are avoided. In addition, the method preserved proteolytic activity and was efficient in removing colored compounds, which simplified further purification and activity determinations. Two cysteine proteinase activities were separated by gel filtration and cation exchange chromatography. PLCP-1 from unbound material was additionally purified on DEAE Sephacel. Finally, both PLCP-1 and PLCP-2 were separately purified by covalent chromatography on Thiopropyl Sepharose at pH = 4.5. This step showed the highest degree of purification for both activities (Table I) and much higher efficiency than in the case of purification of cysteine proteinases from *Phaseolus* leaves, where the binding to Thiopropyl Sepharose was performed at higher pH.[39] The purity of both proteinases was demonstrated by SDS-PAGE, which showed a single band with M_r of 23 400 kDa for PLCP-2 and a doublet with apparently the same molecular mass for PLCP-1.

Their pH optima and pH stability in the slightly acidic region, M_r, and their strong inhibition by E-64, cystatin C and stefin A, rank the two enzymes as typical of the papain family.[5] In mammals, the representatives of this family are usually located in lysosomes and are involved in protein turnover, while vacuoles would be the corresponding location in plant cells.[1,4] Our recent study confirmed the location of PLCP-2 in protein body/vacuoles of the leaf as well as at some other sites.[12]

In general, potato proteinase inhibitors of the Kunitz type and multicystatin have been considered as part of the defense mechanism directed against external proteinases from pathogens which could act during pathogen penetration or are released after mechanical wounding.[19,24,40] In this study, we demonstrate the efficiency of inhibition of cysteine proteinases PLCP-1 and PLCP-2 by different PCPIs and by multicystatin, indicating their possible endogenous function. The pronounced selectivity of different PCPIs for target enzymes and a comparison with inhibition of papain as a model proteinase have been shown as well. Further, the competitive nature of their inhibition of cysteine proteinases was established. Among the PCPIs, the strongest inhibitor of PLCP-1 and PLCP-2 was PCPI 9.4, followed by PCPI 8.3. PCPI 6.6 and PCPI 5.4 were less effective, with K_is in the 10^{-6} M range. Multicystatin was a stronger inhibitor of both proteinases by up to 3 orders of magnitude. It reacted with active PLCP-1 and PLCP-2 in stoichiometry of 1:4. Walsh and Strickland determined a stoichiometry of 1:8 for the multicystatin : papain binding,[24] but their calculations were based on the protein concentration of papain, assuming that no active enzyme is required for interaction. The currently known localization of PCPI 6.6, PCPI 9.4 and multicystatin indicates the possibility of interaction with PLCP-2 *in vivo*.[25] On the basis of K_is, according to Bieth,[41] PCPI 6.6, PCPI 9.4 and multicystatin at concentrations of about 10 μmol dm^{-3}, 0.6 μmol dm^{-3} and 4 nmol dm^{-3}, respectively, could be of physiological significance in inhibiting PLCP-1 or PLCP-2 *in vivo*.

Characterization of both cysteine proteinases PLCP-1 and PLCP-2 revealed their great similarity to each other as well as to cysteine proteinase from sprouting potato tubers.[7] Their physiological substrates and their spatial or temporal expression or activation remain to be established, but the results reported here provide some insight into the possible regulation of their activity by endogenous inhibitors.

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SAŽETAK

Pročišćavanje i karakterizacija dvaju cisteinskih proteinaza iz listova krumpira i način njihove inhibicije endogenim inhibitorima

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Dvije cisteinske proteinaze, PLCP-1 i PLCP-2, pročišćene su iz listova krumpira (*Solanum tuberosum* L.). Elektroforezom PLCP-2 na poliakrilamidnom gelu u prisutnosti natrijevog dodecilsulfata (SDS-PAGE) dobivena je jedna vrpca molekulske mase 23 400, dok je PLCP-2 dala dublet u istom rasponu M_r. Izoelektričnim fokusiranjem PLCP-2 dobivene su dvije vrpce. Jedna je odgovarala pI-vrijednosti 4,6 a druga 4,9. Oba enzima pokazala su pH-optimum i maksimalnu stabilnost pri malo kiselom pH, i snažnu inhibiciju L-*trans*-epoksisukcinoilleucilamido(4-guanidino)butanom (E-64), cistatinom C i stefinom A, što je omogućilo njihovo svrstavanje u porodicu cisteinskih proteinaza. PLCP-1 i PLCP-2 inhibiraju se inhibitorima cisteinskih proteinaza Kunitzovog tipa (PCPI) i multicistatinom, izoliranim iz gomolja krumpira. Najjači inhibitori bili su PCPI 5,4 s K_i od oko 10^{-8} mol dm^{-3}, i PCPI 8,3 s K_i od oko 10^{-7} mol dm^{-3}, dok su K_i-vrijednosti za PCPI 6,6 i PCPI 5,4 bile u mikromolarnom području. Multicistatin se pokazao najmoćnijim inhibitorom obaju proteinaza s K_i-vrijednostima od oko 0,5 nmol dm^{-3}. Stehiometrija kompleksa proteinaza : inhibitor bila je 1:4 za obje proteinaze i multicistatin. Raspravljan je mogući fiziološki značaj ovih endogenih inhibitora, prisutnih i u listovima krumpira. PLCP-1 i PLCP-2 ne mogu se razlikovati na temelju K_i-vrijednosti.