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Proteolysis of the 85-Kilodalton Crystalline Cysteine Proteinase Inhibitor from Potato Releases Functional Cystatin Domains

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The protein crystals found in potato (*Solanum tuberosum* L.) tuber cells consist of a single 85-kD polypeptide. This polypeptide is an inhibitor of papain and other cysteine proteinases and is capable of binding several proteinase molecules simultaneously (P. Rodis, J.E. Hoff [1984] Plant Physiol 74: 907–911). We have characterized this unusual inhibitor in more detail. Titrations of papain activity with the potato papain inhibitor showed that there are eight papain binding sites per inhibitor molecule. The inhibition constant (K_i) value for papain inhibition was 0.1 nM. Treatment of the inhibitor with trypsin resulted in fragmentation of the 85-kD polypeptide into a 32-kD polypeptide and five 10-kD polypeptides. The 32-kD and 10-kD fragments all retained the ability to potently inhibit papain (K_i values against papain were 0.5 and 0.7 nM, respectively) and the molar stoichiometries of papain binding were 2 to 3:1 and 1:1, respectively. Other nonspecific proteinases such as chymotrypsin, subtilisin Carlsberg, thermolysin, and proteinase K also cleaved the 85-kD inhibitor polypeptide into functional 22-kD and several 10-kD fragments. The fragments obtained by digestion of the potato papain inhibitor with trypsin were purified by reverse-phase high-performance liquid chromatography, and the N-terminal amino acid sequence was obtained for each fragment. Comparison of these sequences showed that the fragments shared a high degree of homology but were not identical. The sequences were homologous to the N termini of members of the cystatin superfamily of cysteine proteinase inhibitors. Therefore, the inhibitor appears to comprise eight tandem cystatin domains linked by proteolytically sensitive junctions. We have called the inhibitor potato multicystatin (PMC). By immunoblot analysis and measurement of papain inhibitory activity, PMC was found at high levels in potato leaves (up to 0.6 mg/g fresh weight tissue), where it accumulated under conditions that induce the accumulation of other proteinase inhibitors linked to plant defense. PMC may have a similar defensive role, for example in protecting the plant from phytophagous insects that utilize cysteine proteinases for dietary protein digestion.

The occurrence of discrete proteinaceous crystals within the cells of potato (*Solanum tuberosum* L.) tubers has been well documented (Hoff et al., 1972). The cuboidal crystals are found primarily in the cells of the subphellogen layer of the tuber and are 5 to 25 μm in size. Rodis and Hoff (1984) showed that the isolated crystals consist of a single 85-kD polypeptide that is an effective inhibitor of Cys proteinases, including the plant proteinases papain, ficin, and chymopapain. The potato papain inhibitor is significantly larger than any other plant proteinase inhibitor so far described, and it can simultaneously bind several Cys proteinase molecules. The isolated protein also has an unusual physical property in that it readily crystallizes in mildly basic solutions and in the presence of phosphate. This property can be used to isolate the protein in large amounts from potato peel (Rodis and Hoff, 1984).

Almost all of the well-characterized inhibitors of Cys proteinases described to date are members of a family of inhibitors that are related by both structure and function, called cystatins (Barrett, 1987; Turk and Bode, 1991). The most thoroughly described cystatin is the 13-kD inhibitor from hen egg (Anastasi et al., 1983), although many other cystatins have now been characterized. These have been grouped into three classes (Barrett, 1987): type-1 cystatins, or stefins (M_r about 11,000), are predominantly intracellular proteins and contain no disulfide bonds or associated carbohydrate; type-2 cystatins (M_r about 13,000), such as hen egg cystatin, are typically secreted and contain two disulfide bonds in the C-terminal portion of the polypeptide; type-3 cystatins, or kininogens (M_r 50,000–114,000), are the most complex and comprise three tandemly linked N-terminal type-2 cystatin-like domains that are fused to a vasoactive kinin sequence and a C-terminal extension of varying length (Muller-Esterl et al., 1986). The interaction of cystatins with papain has been defined by the elegant x-ray crystallographic studies of Bode et al. (1988, 1990) and Stubbs et al. (1990). These show that highly conserved residues and secondary structures in the amino acid sequences of cystatins, including the cystatin "signature motif" QXVXG, are part of the intimate contact regions between the inhibitor and target proteinase.

The cystatin classification system and evolutionary relationships (Barrett, 1987; Turk and Bode, 1991) have been derived almost exclusively from analysis of cystatins from vertebrate sources. However, a small number of Cys proteinase inhibitors from plants have now been identified as cystatins, including two inhibitors from rice seeds (oryzacystatins 1 and 2; Kondo et al., 1990) and inhibitors from corn (Abe et al., 1992), soybean (Brzin et al., 1990; Hines et al., 1991), and *Wisteria floribunda* seeds (Hirasaki et al., 1990). The two oryzacystatins and corn cystatin contain no disulfide bonds

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Abbreviations: BAPNA, *N*-α-benzoyl-L-arginine-*p*-nitroanilide; PMC, potato multicystatin; SEC, size-exclusion chromatography; Z-Phe-Arg-NMec, *N*-benzyloxycarbonyl-L-phenylalanyl-L-arginine 7-amido-4-methylcoumarin.

and are therefore stefin-like (type-1 cystatin), but have a greater amino acid sequence homology to type-2 cystatins.

In this study, we show that the 85-kD inhibitor from potato tubers can bind eight papain molecules simultaneously. Treatment of the polypeptide with a range of nonspecific proteinases such as trypsin or subtilisin fragments the inhibitor into a characteristic set of smaller polypeptides (of 10, 22, or 32 kD) that retain the ability to inhibit papain. N-terminal amino acid sequencing of the fragments indicates that they are homologous to the cystatin superfamily of inhibitors. Therefore, we have called the inhibitor PMC. We have also shown by immunoblot analysis that PMC accumulates to high levels in both leaves and tubers as the intact 85-kD form. The various properties of PMC may be linked to a role as a defensive protein against insect and pathogen attack.

MATERIALS AND METHODS

Materials

PMC was purified from the peel of potato (*Solanum tuberosum* L.) tubers purchased from the local market essentially as described by Rodis and Hoff (1984). Typical yields were 10 to 50 mg of pure PMC from 10 lb of potato tubers. After purification, the protein was homogeneous as determined by SDS-PAGE and ion-exchange and reverse-phase HPLC. Hen egg cystatin was purchased from TaKaRa Biochemical, Inc. (Berkeley, CA). Z-Phe-Arg-NMec was purchased from Bachem Inc. (Torrance, CA). All other reagents were purchased from Sigma.

Inhibition of Papain

The stoichiometry of papain inhibition by PMC and hen egg cystatin was measured by determining papain activity with BAPNA as substrate. In the wells of a microtiter plate, 50 μL of inhibitor solution at various concentrations was mixed with 5 μg (214 pmol) of papain in 50 μL of 200 mM sodium acetate, pH 6.0, containing 8 mM DTT and 4 mM sodium EDTA (assay buffer). After 10 min of incubation, 100 μL of 2 mM BAPNA in 100 mM sodium acetate, pH 6.0, was added. The A_{405} was recorded after 1 h using a microplate reader.

K_i values were determined using the papain substrate Z-Phe-Arg-NMec based on the method of Barrett and Kirschke (1981). Fifty nanograms of papain were incubated with 10 to 100 ng of PMC or hen egg cystatin in a total volume of 0.1 mL of assay buffer. Z-Phe-Arg-NMec (0.2 mL) was added to start the reaction. Hydrophobic interactions of PMC with the microtiter plate were eliminated by the addition of 0.01% Triton X-100. The presence of the detergent had no apparent effect on the calculated K_i values. Data were collected using a Labsystems Fluoroskan II fluorescent microtiter plate reader linked to a Biometallics data collection software system with kinetic capabilities. Excitation and emission wavelengths were 380 and 460 nm, respectively. Kinetic assays were performed at inhibitor concentrations, giving approximately 30, 50, and 70% inhibition of papain, and Z-Phe-Arg-NMec concentrations were varied from 1 to 20 μM. A K_m value of 6.2 μM was derived for Z-Phe-Arg-NMec as a papain substrate.

Proteolytic Fragmentation of PMC

Native 85-kD PMC was fragmented by digestion with trypsin (Sigma type XIII). Digestions were performed in 50 mM Tris-Cl, pH 7.5, at 37°C for 2 h with a PMC:trypsin ratio of 20:1 (w/w). Fragmentation was also performed using chymotrypsin (Sigma type VII), subtilisin Carlsberg, proteinase K, and thermolysin using the same conditions. The stoichiometry of papain inhibition by trypsin-treated PMC was measured after the trypsin was inactivated by addition of 3,4-dichloroisocoumarin (100 μM final concentration) to the digestion. Separate experiments showed that 3,4-dichloroisocoumarin had no effect on papain activity.

Chromatography

SEC of PMC and proteinase-treated PMC was performed using Pharmacia Superose 12 columns. Protein standards used to calibrate the columns were from Bio-Rad. For analytical separations (<2 mg), a Superose 12 HR 10/30 column was used at a flow rate of 0.4 mL/min, and, for preparative runs, a Superose 12 16/50 column was used at a flow rate of 1 mL/min. Columns were equilibrated in 20 mM Tris-Cl, pH 8.0, containing 150 mM sodium chloride. Lower salt concentrations gave poor separations due to interactions of the PMC fragments with the column matrix. Separations at pH 5.0 were performed with 0.2 M sodium acetate as buffer.

Reverse-phase chromatography was performed using a Vydac C_4 column equilibrated in 0.1% TFA. Separation of polypeptides derived from PMC after trypsin treatment was done at a flow rate of 1 mL/min using a gradient of 0 to 40% acetonitrile over 2 min, then a 40 to 54% acetonitrile gradient over 60 min.

Amino Acid Sequencing

N-terminal amino acid sequencing of purified PMC and the fragments purified by reverse-phase HPLC was performed by Dr. William S. Lane at the Harvard Microchemistry Facility (Cambridge, MA). Sequences were determined using an Applied Biosystems 470A gas phase sequencer with an on-line phenylthiohydantoin amino acid analyzer. The yield of N-terminal phenylthiohydantoin amino acids from native PMC and PMC-10–2 was extremely low, suggesting that the N-termini were resistant to Edman degradation. To obtain amino acid sequence data for PMC-10–2, the polypeptide was denatured in urea and digested for 17 h with trypsin. The resulting digestion was separated using a Vydac C_{18} column. Peptides in two of the resulting peaks were sequenced (PMC-10–2:T51 and PMC-10–2:T32). The sequence data shown in Figure 5 suggest that some residual chymotryptic activity was present in the digestion.

PAGE

Routine SDS-PAGE and IEF were performed with a Pharmacia Phast System. Homogeneous 20 Phastgels were used for SDS-PAGE and IEF 3–9 Phastgels were used for IEF.

Analysis of Leaf Extracts

Leaves were taken from greenhouse- or field-grown potatoes and were either frozen immediately at −70°C or induced for 3 d as described by Akers and Hoff (1980). The leaves were homogenized in 0.2 M sodium acetate, pH 6.0, containing 10 μM 3,4-dichloroisocoumarin. After clarification by centrifugation, the amount of inhibitory activity in the extracts was determined by titration of papain as described above. For immunoblot analysis, tissues were extracted in 2 volumes of 150 mM Tris-Cl, pH 7.8, containing 150 μg/mL leupeptin, 150 μg/mL antipain, and 6 mM sodium EDTA. After denaturation in SDS, samples were electrophoresed in 12.5% SDS-PAGE gels (Integrated Separation Systems, Natick, MA) and transferred onto Millipore Immobilon-P paper using a Pharmacia LKB Transphor apparatus. The blots were probed with rabbit polyclonal antiserum against undenatured, 85-kD PMC at a 1:2000 dilution prepared by the Berkeley Antibody Company (Berkeley, CA). The blots were developed with alkaline phosphatase-linked goat anti-rabbit antibody using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium from Bio-Rad as substrates according to the supplier's instructions. The intensity of the 85-kD bands was compared with standards containing known amounts of PMC purified from tubers.

Protein Concentrations

Protein concentrations were determined by the method of Bradford (1976). Concentrations of purified PMC solutions were determined using A_{280} = 1.2 mL mg^{-1} cm^{-1} (Rodis and Hoff, 1984).

RESULTS

Stoichiometry of Papain Inhibition by PMC

Papain was titrated with PMC and the residual proteinase activity at pH 6.0 was monitored by a spectrophotometric assay using BAPNA as substrate. These titrations consistently showed that 8 (±0.14) mol of papain were inhibited by 1 mol of PMC (Fig. 1). This stoichiometry was reproduced with several different batches of PMC and papain. Titration of papain activity at pH 8 also gave the same stoichiometry. Similar titrations using hen egg cystatin, a well-characterized, single-domain polypeptide inhibitor of Cys proteinases (Anastasi et al., 1983), gave the expected 1:1 molar stoichiometry. Therefore, we conclude that there are eight binding domains for papain within each PMC polypeptide.

Fragmentation of PMC by Limited Proteolysis

Treatment of native 85-kD PMC with trypsin gave rise to a distinct and reproducible fragmentation pattern by SDS-PAGE analysis. This pattern consisted of a band at 32 kD and a family of smaller molecular mass bands of approximately 10 kD (Fig. 2). There was also a fainter band at 22 kD. This pattern was stable even after prolonged incubation with trypsin (3 h at 37°C) using PMC:trypsin ratios of up to 20:1 (w/w). The final fragmentation pattern was arrived at via a characteristic series of larger intermediates (data not shown), indicating a sequential degradation process. The stoichiometry of papain inhibition by PMC after treatment with trypsin was identical to that of the untreated inhibitor (Fig. 1), demonstrating that proteolytic fragmentation did not destroy the ability of any of the domains to bind papain. Trypsin-treated PMC did not crystallize under the conditions in which the native protein readily crystallized, i.e. at basic pH and/or in the presence of phosphate. Indeed, a suspension of crystalline PMC at pH 8 was readily solubilized by addition of trypsin.

Other diverse and relatively nonspecific proteinases such as chymotrypsin, subtilisin Carlsberg, thermolysin, and proteinase K also cleaved PMC into a similar fragmentation pattern of polypeptides. In these cases, the intensity of the larger fragments was reversed and a pronounced band at 22 kD and a relatively faint band at 32 kD were observed by SDS-PAGE in addition to the bands at 10 kD (Fig. 2). These fragments also retained their ability to effectively inhibit papain in titrations.

SEC of Trypsin-Treated PMC

SEC of native PMC at pH 8 using a Pharmacia Superose 12 column resulted in a predominant peak corresponding to a molecular mass of approximately 360 kD, suggesting that PMC is a tetramer at this pH (Fig. 3A). A minor peak of approximately 100 kD was also noted. At pH 5, PMC eluted from the Superose 12 column with a retention time that corresponded to a molecular mass of approximately 55 kD (data not shown), indicating that PMC is monomeric at this pH and in agreement with previous observations by Rodis and Hoff (1984). SEC of trypsin-treated PMC at pH 8 yielded two peaks with increased retention times (Fig. 3B). Analysis by SDS-PAGE showed that the first peak contained the 32-kD polypeptide (and a smaller amount of 22-kD fragment)

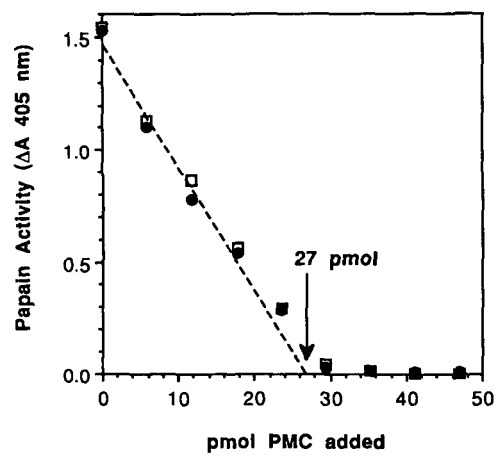

Figure 1. Stoichiometric titration of papain with PMC. The proteinase activity of 5 μg (214 pmol) of papain was monitored using BAPNA as substrate in the presence of increasing amounts of PMC. The extrapolated intersect on the x axis (27 pmol of PMC) corresponds to a papain:PMC molar ratio of 7.9:1. Titrations were performed with native 85-kD PMC (●) or PMC after treatment with trypsin (□) as described in "Materials and Methods."

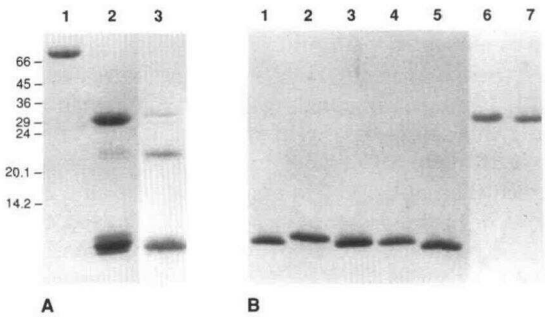

Figure 2. SDS-PAGE analysis of PMC after digestion with proteinases. A, Lane 1, 0.4 μg of purified 85-kD PMC; lane 2, 1.5 μg of PMC after treatment with trypsin as described in "Materials and Methods"; lane 3, 0.5 μg of PMC after treatment with subtilisin. B, Six hundred micrograms of trypsin-treated PMC was separated by reverse-phase HPLC as described in "Materials and Methods." The resulting peaks (shown in Fig. 4) were collected, lyophilized, and analyzed by SDS-PAGE. Lanes 1–5, PMC-10–1 through PMC-10–5; lane 6, PMC-33; lane 7, PMC-32. Positions of molecular mass standards (kD) for both panels are shown on the left.

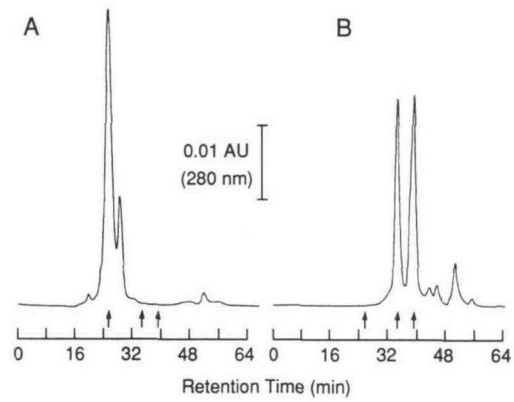

Figure 3. SEC of PMC. Samples of PMC were applied to a Superose 12 10/30 column equilibrated in 50 mM Tris-Cl, pH 7.8, containing 100 mM sodium chloride at a flow rate of 0.4 mL/min. A_{280} was monitored. A, Fifty micrograms of native 85-kD PMC; B, 50 μg of PMC after treatment with trypsin as described in "Materials and Methods." The arrows denote the relative positions of the three major peaks.

and the second peak contained the family of 10-kD polypeptides. The retention times of these peaks corresponded to molecular masses of 35 and 12 kD, which is in reasonable agreement with the values derived from SDS-PAGE analysis. Thus, there was no indication of significant association of the PMC fragments at pH 8, in contrast to the native protein.

SEC was a convenient method of obtaining pure preparations of the 32- and 10-kD polypeptides (named PMC-32 and PMC-10, respectively) under nondenaturing conditions. The stoichiometries for papain inhibition by PMC-10 and PMC-32 were 1 mol papain/mol PMC-10 and 2 to 3 mol papain/mol PMC-32 (data not shown). K_i values of the PMC-32 and PMC-10 preparations were determined against papain and compared with that of 85-kD PMC using a sensitive fluorometric assay of papain with Z-Phe-Arg-NMec as substrate. K_i values for 85-kD PMC, PMC-32, and PMC-10 were 0.1 (±0.01), 0.7 (±0.02), and 0.5 (±0.02) nM, respectively.

N-Terminal Amino Acid Sequences of PMC Fragments

The PMC fragments derived by trypsin treatment were readily separated by reverse-phase HPLC (Fig. 4). This separation yielded five major peaks corresponding to the 10-kD polypeptides (PMC-10–1 through PMC-10–5) and an additional major and minor peak at a later retention time (PMC-32 and PMC-33, respectively), corresponding to the 32-kD polypeptides. The purified 10-kD polypeptides were slightly different in size by SDS-PAGE, which accounted for the slight heterogeneity of this band in the gels of trypsin-treated PMC run prior to separation (Fig. 2). The peaks were individually collected and the N-terminal amino acid sequences of the polypeptides determined (Fig. 5A). The PMC-10 fragments were particularly amenable to sequencing, and over 60 residues of sequence data were obtained for PMC-10–4 and PMC-10–5. The N termini of both native PMC and PMC-10–2 were resistant to Edman degradation, indicating that both may be blocked. PMC-10–2 is therefore likely to be the N-terminal fragment of PMC. To obtain amino acid sequence data for PMC-10–2, a sample of the purified polypeptide was denatured in urea to facilitate complete digestion by trypsin. The resulting mixture of oligopeptides was separated by reverse-phase HPLC, and the amino acid sequences of two of the peptides were determined (PMC-10–2:T51 and PMC-10–2:T32 in Fig. 5).

Comparison of the amino acid sequences of the PMC

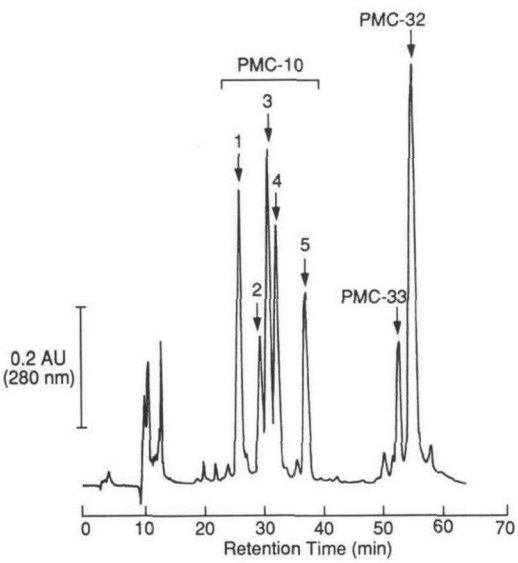

Figure 4. Reverse-phase HPLC of PMC after treatment with trypsin. PMC (400 μg) was digested with trypsin and applied to a Vydac C_4 column as described in "Materials and Methods." The gradient was initiated after 4 min, and A_{280} was monitored. The first five major peaks (25- to 40-min retention times) contained 10-kD polypeptides, and the final two peaks (50- to 60-min retention time) contained polypeptides of approximately 33 and 32 kD by SDS-PAGE analysis (Fig. 2B).

fragments (Fig. 5A) revealed that all of the sequences showed an appreciable degree of homology with each other. Indeed, PMC-10–4 and PMC-10–5 shared 75% homology, which extended through the entire 61 residues of sequence information that was obtained. Further analysis of the sequences derived from the trypsin-treated PMC polypeptides showed that they were all related to the cystatin superfamily of Cys proteinase inhibitors (Barrett, 1987; Turk and Bode, 1991). This is apparent from the comparison in Figure 5B of the N-terminal amino acid sequence of PMC-10–5 with those of oryzacystatin 1 from rice and hen egg cystatin (Turk et al., 1983; Schwabe et al., 1984; Abe et al., 1987). There is a significant degree of homology with both polypeptides throughout the sequences (44 and 28% identity, respectively). Moreover, the characteristic motif QXVXG that is highly conserved within all cystatins (Barrett, 1987; Turk and Bode, 1991) is found in PMC-10–4, PMC-10–5, and PMC-10–2:T51. Cystatins also possess a conserved Gly residue in the N-terminal region of the polypeptide (Gly^9 of hen egg cystatin and Gly^4 of oryzacystatin 1). An equivalent Gly residue is found at the second or third position in all of the sequenced PMC fragments.

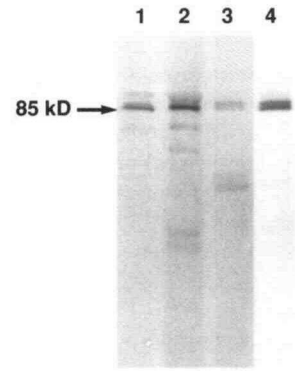

Figure 6. Immunoblot analysis of protein extracts from potato plant tissues. Samples were subjected to SDS-PAGE and blots were probed with anti-85-kD PMC serum as described in "Materials and Methods." Lane 1, Ten nanograms of PMC purified from tuber peel; lane 2, tuber peel extract (10 μg of total protein); lane 3, uninduced leaf tissue extract (10 μg of total protein); lane 4, leaf tissue extract (2.4 μg of total protein) after induction for 3 d as described by Akers and Hoff (1980).

Immunoblot Analysis of PMC Expressed in Potato Leaves and Tuber

Akers and Hoff (1980) correlated the formation of cubical crystals with an increase in inhibitory activity against chymopapain in the leaves of tomato seedlings after leaf excision and continuous illumination. These conditions have been shown to induce accumulation of wound-responsive proteinase inhibitors such as Ser proteinase inhibitors I and II, metallo-carboxypeptidase inhibitor, and Kunitz-type Ser proteinase inhibitors (Ryan, 1990; Suh et al., 1991). Also, Hildmann et al. (1992) have recently shown that a cDNA with homology to oryzacystatin is induced by mechanical wounding and ABA treatment. We performed experiments similar to those of Akers and Hoff (1980) with excised potato leaves from greenhouse- and field-grown plants and analyzed the resulting tissue extracts both by immunoblot using a polyclonal antiserum raised against native 85-kD PMC and by measuring the amount of papain inhibitory activity in the extracts. A group of three bands at approximately 85 kD was recognized in immunoblots of the leaf tissue extracts, whereas extracts from tuber skin had two major 85-kD bands, with the lower band being more prominent (Fig. 6). No evidence for major accumulation of smaller M_r fragments was noted, although minor amounts of smaller fragments were visualized in the immunoblots. However, these were reduced using rapid extraction procedures in the presence of protease inhibitors, so they are likely due to a low level of proteolysis during extraction. Some PMC could be detected in uninduced leaf samples (Fig. 6, lane 3; 10 μg of total protein loaded), but the amount was significantly enhanced after induction (Fig. 6, lane 4; 2.4 μg of total protein loaded). Comparison of the

(A)

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PMC-10-1          FGGIIS
PMC-10-2:T51                                              QQIVAGIMY
PMC-10-2:T32                                                      YITFEATEGGK
PMC-10-3          LGGIVNVPNPNN
PMC-10-4          FGGIISVPFPNSPEFQDLARFAVQDYNNTQNAHLEFVENLSVKEQLVSGMMYYITLAATXS
PMC-10-5          YGGLTDVPFFNNPEFQDLARFAVQDYNKKENGHLEFVEVLNVKEQVVAGMMYYITLAATXVG
PMC-32           IPGGFTEVPFFPN
PMC-33           IPGGFTEVPFFPN
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(B)

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                   *                                                  * * *
OC-1          MSSDGGPVLGGVEPVGNENDLHLVDLARFAVTEHNKKANSLL--EFEKLVSVKQQVVAGTLYYFTIEVKEG...
                   ||:  |    |     |||||||  : |    |  |  ||   :||| |:| |  || |:
PMC-10-4           FGGIISVPFPNSPEFQDLARFAVQDYNNTQNAHL--EFVENLSVKEQLVSGMMYYITLAATXS
                   |: : |    |     |     ||  :||   |       |  :| | |||||  | : :
HEC           SEDRSRLLGAPVPV-DENDEGLQRALQFAMAEYNRASNDKYSSRVVRVISAKRQLVSGIKYILQVEIGRT...
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Figure 5. Partial amino acid sequences of fragments derived from trypsin treatment of PMC. A, N-terminal sequences of PMC fragments from the peaks in Figure 4. PMC-10–2 was refractory to Edman degradation and so was further digested with trypsin as described in "Materials and Methods" to give the oligopeptides PMC-10–2:T51 and PMC-10–2:T32, which were completely sequenced. Underlined residues are lower-confidence assignments. B, Amino acid sequence alignment of PMC-10–4 with oryzacystatin 1 (OC-1; Abe et al., 1987) and hen egg cystatin (HEC; Turk et al., 1983; Schwabe et al., 1984). Vertical lines (|) denote identical residues and colons (:) denote homologous substitutions. Highly conserved residues of cystatins, i.e. the Gly residue corresponding to Gly^9 of hen egg cystatin and the motif QXVXG, are marked with asterisks (*).

intensity of the major 85-kD bands with that of known amounts of purified tuber PMC indicated that levels of up to 2% total soluble leaf protein or 300 to 600 μg PMC/g fresh weight of leaf were present in the leaves after induction.

Titrations of papain with protein extracts from "induced" leaves from the field showed that there was an increase in papain inhibitory activity over that in uninduced leaves. Based on the levels of papain inhibition, induced leaves from field-grown plants were estimated to contain 1.6% of the total soluble leaf protein as PMC, in good agreement with the immunoblot data. Therefore, PMC appears to accumulate in potato leaves under conditions similar to those in which other tuber inhibitors of proteinases accumulate. Immunoblots in similar experiments using tomato leaves show a band at 85 kD of reduced intensity relative to that in the potato leaf extracts. This may be due to lower amounts of a PMC analog being produced, or because of reduced cross-reactivity of the homologous polypeptide from tomato with the antiserum raised against the inhibitor from potato.

DISCUSSION

The size of most characterized plant proteinase inhibitors is in the range of 8 to 25 kD (Garcia-Olmedo et al., 1987; Ryan, 1990). Therefore, PMC is unusually large at 85 kD, suggesting that it may contain several proteinase-binding sites. Indeed, Rodis and Hoff (1984) noted that the polypeptide bound four papain molecules. However, our titrations of papain with PMC consistently indicate that there are eight papain-binding sites per PMC polypeptide. We have obtained the same 8:1 stoichiometry at pH 6 and 8, with different preparations of PMC and papain. To further confirm our stoichiometry, we performed similar titrations with the well-characterized, single-domain cystatin from hen egg. These gave the expected 1:1 molar stoichiometry.

We have found that treatment of PMC under native conditions with trypsin, chymotrypsin, subtilisin Carlsberg, proteinase K, and thermolysin will cleave PMC into smaller functional polypeptides, and it seems likely that a wide range of nonspecific proteinases (other than those Cys proteinases that are inhibited by PMC) will readily process the 85-kD PMC polypeptide. Although inhibitory activity is maintained, the ability of PMC to readily crystallize in basic conditions is lost after proteolysis. Reverse-phase HPLC analysis of trypsin-treated PMC reveals that the resulting polypeptides consist of five approximately 10-kD fragments and a major 32-kD fragment that are quite stable to further trypsin treatment once formed. The 32-kD fragment binds two to three papain molecules, whereas the 10-kD polypeptides have a 1:1 stoichiometry. The N-terminal amino acid sequence data for these fragments show that the polypeptides are similar but not identical, and that they are related to the cystatin superfamily of Cys proteinase inhibitors. These data indicate that PMC is a multidomain polypeptide consisting of eight tandem cystatin domains (of approximately 10 kD) that are linked by proteolytically sensitive junctions. This accounts for the large molecular mass of PMC, the stoichiometry of papain inhibition, and the proteolytic fragmentation into functional domains. We have called this type of Cys protease inhibitor a "multicystatin." There may be other multicystatins within the plant kingdom; for example, a homolog of PMC is produced in leaflets of tomato, a related solanaceous plant (Akers and Hoff, 1980; this work).

There are many examples of proteinase inhibitors that have more than one proteinase-binding site within a single polypeptide chain. For example, the Bowman-Birk inhibitors of Ser proteinases from plants are double-headed and can inhibit trypsin and chymotrypsin simultaneously in the case of the lima bean inhibitor (Garcia-Olmedo et al., 1987). Within the vertebrates, the plasma lipoprotein-associated coagulation inhibitor consists of three tandem Kunitz-type inhibitor domains (Wun et al., 1988), the avian ovomucoid and ovoinhibitor families have members containing up to seven Kazal-type inhibitor domains (Kato et al., 1987; Scott et al., 1987), and the plasma kininogens contain three cystatin-like domains (Muller-Esterl et al., 1986). Only two of the three cystatin-like domains of kininogens appear to be functional Cys proteinase inhibitors (Muller-Esterl et al., 1986), whereas in PMC, all the domains appear to be active.

PMC and the kininogens are similar in that they have regions between cystatin domains that are sensitive to cleavage by the action of a variety of proteinases, and processing at these sites results in the release of active domains (Vogel et al., 1988; Moreau et al., 1989). The interdomain regions of the avian Kazal inhibitors are similarly proteolytically sensitive (Scott et al., 1987). In the plant kingdom, a recently described cDNA from *Nicotiana alata* stigmas encodes a 42-kD polypeptide containing six proteinase inhibitor II-type domains (Atkinson et al., 1993). However, this polypeptide appears to function as a secreted precursor protein that is specifically cleaved in vivo, leading to accumulation of the individual 6-kD domains. In contrast, our immunoblot analyses of potato leaves and tubers indicate that the predominant accumulated form of PMC is the intact 85-kD polypeptide.

We have found no evidence for cooperativity between the PMC-binding sites in analysis of papain titrations or K_i determinations. This implies that the eight sites on PMC are always freely accessible to papain. The eight cystatin domains within PMC must therefore be structurally arranged so that the regions that bind Cys proteinases such as papain as well as the interdomain regions susceptible to cleavage by processing proteinases such as trypsin are exposed. The interdomain regions are not all completely equivalent or accessible to processing proteinases, because a stable 32-kD fragment is formed after PMC digestion with trypsin that is refractory to further trypsin digestion, with only a trace of 22-kD polypeptide being formed. Digestion by the other proteinases that we have tested leads to a 22-kD (rather than a 32-kD) species, in addition to the single-domain 10-kD forms. This may be a function of sequence differences in the interdomain regions or differences in presentation or accessibility of specific regions to the processing proteinases.

The amino acid sequences of the PMC fragments are similar but not identical. It is possible that some of the heterogeneity in sequences that we have obtained may be due to the presence of different isoforms of PMC in the tuber. We believe that this is not a large source of the sequence heterogeneity because our preparations of the intact 85-kD PMC polypeptide behave quite uniformly. For example, analysis of the purified native protein gives a single major

band in IEF gels, and a single symmetrical peak is obtained by Mono-Q anion-exchange chromatography and reverse-phase HPLC. However, after proteolytic fragmentation several bands are seen in IEF gels, and several peaks can be separated by both methods of chromatography. These data are consistent with there being differences among the sequences of the eight cystatin domains contained within the 85-kD PMC, rather than there being a large number of different 85-kD PMC isoforms.

The various unique properties of PMC may be linked to a specific role as a defensive protein against insect and pathogen attack based on the following considerations:

(a) PMC crystals are produced mainly in the peridermal cells of tubers (Hoff et al., 1972). In this location, they would be in the first line of defense against pathogen and insect attack. PMC is also wound-inducible in leaf tissue, where we have shown that it can accumulate to high levels (0.6 mg/g fresh weight leaf tissue), in common with other proteinase inhibitors in potato that have been associated with plant defense (Ryan, 1990; Suh et al., 1991).

(b) Many phytophagous coleopteran insect larvae utilize Cys proteinases as the major digestive proteinases of the midgut (Murdock et al., 1987). We have shown that growth of such insects can be significantly suppressed by the presence of PMC in the diet at levels equivalent to those found in vivo (G. Orr, M. Arrington, J. Strickland, and T. Walsh, unpublished data).

(c) The crystals are solubilized at low pH (Rodis and Hoff, 1984) and by proteinase treatment (this work). Insects with Cys proteolytic midguts typically have mildly acidic gut environments (Murdock et al., 1987) that would solubilize the crystals. Also, many invading microbial pathogens typically secrete proteinases that could solubilize the crystals and result in inhibition of any Cys proteinase components involved in pathogen attack.

(d) The inhibitor crystals are cytosolic (shown well in micrographs of Boulter and Harvey, 1985) and so are in a cellular compartment away from endogenous Cys proteinases that are commonly found in vacuolar and extracellular compartments of plant tissue and that could potentially bind to and neutralize the inhibitory activity of an accumulating Cys proteinase inhibitor.

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LITERATURE CITED

Abe M, Abe K, Kuroda M, Arai S (1992) Corn kernel cysteine protease inhibitor as a novel cystatin superfamily member of plant origin. Eur J Biochem **209:** 933–937

Abe K, Emori Y, Kondo H, Suzuki K, Arai S (1987) Molecular cloning of a cysteine proteinase inhibitor from rice (oryzacystatin). J Biol Chem **262:** 16793–16797

Akers CP, Hoff JE (1980) Simultaneous formation of chymopapain inhibitor activity and cubical crystals in tomato leaves. Can J Bot **58:** 1000–1003

Anastasi A, Brown MA, Kembhavi AA, Nicklin MJH, Sayers CA, Sunter DC, Barrett AJ (1983) Cystatin, a protein inhibitor of cysteine proteases. Improved purification from egg white, characterization, and detection in chicken serum. Biochem J **211:** 129–138

Atkinson AH, Heath RL, Thompson RJ, Clarke AE, Anderson MA (1993) Proteinase inhibitors in *Nicotiana alata* stigmas are derived from a precursor protein which is processed into five homologous inhibitors. Plant Cell **5:** 203–213

Barrett AJ (1987) The cystatins: a new class of peptidase inhibitors. Trends Biochem Sci **12:** 193–196

Barrett AJ, Kirschke H (1981) Cathepsin B, cathepsin H and cathepsin L. Methods Enzymol **80:** 535–561

Bode W, Engh R, Musil D, Laber B, Stubbs M, Huber R, Turk V (1990) Mechanism of interaction of cysteine proteases and their protein inhibitors as compared to the serine proteinase-inhibitor interaction. Biol Chem Hoppe-Seyler Suppl **371:** 111–118

Bode W, Engh R, Musil D, Thiele U, Huber R, Karshikov A, Brzin J, Kos J, Turk V (1988) The 2.0 Å x-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteases. EMBO J **7:** 2593–2599

Boulter D, Harvey PJ (1985) Accumulation, structure and utilisation of tuber storage proteins with particular reference to *Dioscorea rotundata*. Physiol Veg **23:** 61–74

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brzin J, Ritonja A, Popovic T, Turk V (1990) Low molecular mass protein inhibitor of cysteine proteinases from soybean. Biol Chem Hoppe-Seyler Suppl **371:** 167–170

Garcia-Olmedo F, Salcedo G, Sanchez-Monge R, Gomez L, Royo J, Carbonero P (1987) Plant proteinaceous inhibitors of proteinases and α-amylases. Oxf Surv Plant Mol Cell Biol **4:** 275–334

Hildmann T, Ebneth M, Pena-Cortes H, Sanchez-Serrano JL, Willmitzer L (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4:** 1157–1170

Hines ME, Osuala CI, Nielsen SS (1991) Isolation and partial characterization of a soybean cystatin cysteine protease inhibitor of coleopteran digestive proteolytic activity. J Agric Food Chem **39:** 1515–1520

Hirasaki I, Ogata F, Yoshida N, Makisumi S, Ito A (1990) Purification and complex formation analysis of a cysteine protease inhibitor (cystatin) from seeds of *Wisteria floribunda*. J Biochem **108:** 604–608

Hoff JE, Jones CM, Sosa MP, Rodis P (1972) Naturally occurring protein crystals in the potato: isolation and identification as a protein. Biochem Biophys Res Commun **49:** 1525–1529

Kato I, Schrode J, Kohr WJ, Laskowski M (1987) Chicken ovomucoid: determination of its amino acid sequence, determination of the trypsin reactive site, and preparation of all three of its domains. Biochemistry **26:** 193–201

Kondo H, Abe K, Nishimura I, Watanabe H, Emori Y, Arai S (1990) Two distinct cystatin species in rice seeds with different specificities against cysteine proteinases. Molecular cloning, expression and biochemical studies on oryzacystatin-II. J Biol Chem **265:** 15832–15837

Moreau T, Gutman N, Faucher D, Gauthier F (1989) Limited proteolysis of T-kininogen (thiostatin). Release of comparable fragments by different endopeptidases. J Biol Chem **264:** 4298–4303

Muller-Esterl W, Iwanaga S, Nakanishi S (1986) Kininogens revisited. Trends Biochem Sci **11:** 336–339

Murdock L, Brookhart G, Dunn PE, Foard DE, Kelley S, Kitch L, Shade RE, Shukle RH, Wolfson JL (1987) Cysteine digestive proteinases in Coleoptera. Comp Biochem Physiol **87B:** 783–787

Rodis P, Hoff JE (1984) Naturally occurring protein crystals in the potato. Inhibitor of papain, chymopapain, and ficin. Plant Physiol **74:** 907–911

Ryan CA (1990) Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Phytopathol **28:** 425–449

Schwabe C, Anastasi A, Crow H, McDonald JK, Barrett AJ (1984)

Cystatin amino acid sequence and possible secondary structure. Biochem J **217:** 813–817

Scott MJ, Huckaby CS, Kato I, Kohr WJ, Laskowski M, Tsai M-J, O'Malley BW (1987) Ovoinhibitor introns specify functional domains as in the related and linked ovomucoid gene. J Biol Chem **262:** 5899–5907

Stubbs MT, Laber B, Bode W, Huber R, Jerala R, Lenarcic B, Turk V (1990) The refined 2.4 Å x-ray crystal structure of recombinant human stefin B in complex with the cysteine protease papain: a novel type of proteinase inhibitor interaction. EMBO J **9:** 1939–1947

Suh S-G, Stiekema WJ, Hannapel DJ (1991) Proteinase-inhibitor activity and wound-inducible gene expression of the 22-kDa potato-tuber proteins. Planta **184:** 423–430

Turk V, Bode W (1991) The cystatins: protein inhibitors of cysteine proteases. FEBS Lett **285:** 213–219

Turk V, Brzin J, Longer M, Ritonja A, Eropkin M, Borchart W, Machleidt W (1983) Protein inhibitors of cysteine proteinases 3. Amino acid sequence of cystatin from egg white. Hoppe-Seyler's Z Physiol Biochem **364:** 1487–1496

Vogel R, Assfalg-Machleidt I, Esterl A, Machleidt W, Muller-Esterl W (1988) Proteinase-sensitive regions in the heavy chain of low molecular weight kininogen map to the inter-domain junctions. J Biol Chem **263:** 12661–12668

Wun T-C, Kretzmer KK, Girard TJ, Miletich JP, Broze GJ (1988) Cloning and characterization of a cDNA coding for the lipoprotein-associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains. J Biol Chem **263:** 6001–6004