Processing of thionin precursors in barley leaves by a vacuolar proteinase

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Thionins are synthesized as precursors with a signal peptide and a long C-terminal acidic peptide that is post-translationally processed. A fusion protein including the maltose-binding protein from *Escherichia coli* (MalE), thionin DG3 from barley leaves, and its acidic C-terminal peptide has been used to obtain antibodies that recognize both domains of the precursor. In barley leaf sections, mature thionins accumulated in the vacuolar content, while the acidic peptide was not detected in any cell fraction. Brefeldin A and monensin inhibited processing of the precursor but its export from the microsomal fraction was not inhibited. Both purified vacuoles and an acid (pH 5.5) extract from leaves processed the fusion protein into a MalE-thionin and an acidic peptide fragment. A 70-kDa proteinase that effected this cleavage was purified from the acid extract. Processing of the fusion protein by both lysed vacuoles and the purified proteinase was inhibited by Zn^{2+} and by Cu^{2+}, but not by inhibitors of the previously described vacuolar processing thiol or aspartic proteinases. *In vivo* processing of the thionin precursor in leaf sections was also inhibited by Zn^{2+} and Cu^{2+}. Variants of the fusion protein with altered processing sites that represented those of thionin precursors from different taxa were readily processed by the proteinase, whereas changing the polarity of either the C-terminal or N-terminal residues of the processing site prevented cleavage by the proteinase.

Keywords: barley; processing proteinase; thionin; vacuole.

In plant cells, vacuoles play both general and specialized roles that are mediated by proteins which are transported as precursors into these organelles (see reviews by Chrispeels and Raikhel, 1992; Nakamura and Matsuoka, 1993). The proteolytic processing of these precursors has been most extensively studied in the case of seed proteins that are transported into specialized storage vacuoles. Thus, the vacuolar processing activities that convert proglobulin into 11S globulin and pro2S albumin into its mature form in pumpkin cotyledons (Hara-Nishimura et al., 1985, 1993; Hara-Nishimura and Nishimura, 1987), proricin into ricin in castor bean seeds (Harley and Lord, 1985), and proglycinin into glycinin in soybeans seeds (Scott et al., 1992) have been investigated. The processing of sporamin precursors in tubers of the sweet potato (Matsuoka and Nakamura, 1991) and of non-storage vacuolar proteins, such as probarley lectin in leaves (Runeberg-Roos et al., 1994) and barley proaleurain in aleurone (Holwerda et al., 1990), have also been studied.

Hara-Nishimura et al. (1991) purified a processing enzyme from castor bean endosperm that has been characterized at the molecular level and found to be related to a putative cysteine proteinase from *Schistosoma mansoni* (Hara-Nishimura et al., 1993). This enzyme can process a wide range of precursors at the C-terminal side of exposed asparagine residues present on hydrophilic surfaces of the proproteins (Hara-Nishimura et al., 1991, 1993). Similarly, a thiol proteinase is thought to be involved in the second step of proaleurain processing in barley, but not in a first step (Holwerda et al., 1990). Cleavage of proglycinin in soybean seeds also occurs at a conserved Asn-Gly (P1-P1′) site; the enzyme involved was at least partially affected by inhibitors of thiol proteinases, such as *trans*-epoxysuccinyl-L-leucylamido-(4-guanidinio)butane (E64) and $HgCl_2$ (Scott et al., 1992).

A second type of vacuolar processing activity was found to correspond to an aspartic proteinase (D'Hondt et al., 1993; Runeberg-Roos et al., 1994). It has been determined that 13 amino acid residues at the C terminus of probarley lectin are specifically cleaved *in vitro* by a vacuolar aspartic proteinase that is co-localized with the lectin in root cells of developing embryos and germinating barley seedlings (Runeberg-Roos et al., 1994). Previously, it had been shown that an aspartic proteinase present in seeds specifically cleaved *Arabidopsis* 2S albumin precursors *in vitro* (D'Hondt et al., 1993).

Thionins are widely distributed cysteine-rich plant polypeptides that are toxic to plant microbial pathogens and might be involved in redox regulation (see reviews by García-Olmedo et al., 1989, 1992). The five thionin types that have been described have a common precursor structure with a signal peptide at the N terminus and a long acidic peptide at the C terminus, as deduced from their cloned complementary and genomic DNAs (Bohlmann and Apel, 1987; Castagnaro et al., 1992; Gausing, 1987; Hernández-Lucas et al., 1986; Ponz et al., 1986; Rodríguez-Palenzuela et al., 1988; Schrader and Apel, 1991). The study of the *in vitro* and *in vivo* synthesis of type-I thionins from barley endosperm showed that at least two processing steps were required to yield the mature protein, the co-translational cleavage of a signal peptide and the post-translational cleavage of a larger peptide (Ponz et al., 1983). Precursor structures deduced from subsequently determined nucleotide sequences of type-I cDNAs were congruent with these observations (Hernández-Lucas et al., 1986; Ponz et al., 1986). This type of thionin

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Abbreviations. MalE, maltose-binding protein from *Escherichia coli*; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidinio) butane; PhMeSO$_2$F, phenylmethylsulfonyl fluoride; P1 and P1′, C-terminal and N-terminal residues of the processing site.

has been located in electron-dense ovoidal structures present in the periphery of protein bodies in barley and wheat endosperm (Carmona et al., 1993), but the location of the post-translational processing activity is not known. Although the processing of the other thionin types has not been investigated, a common mechanism can be inferred from the highly conserved features of the different precursors. Type-II thionins, which are expressed in barley leaves, were originally thought to be mainly located in the cell wall (Bohlmann et al., 1988), but it was later reported that over 98% of them were in the vacuoles (Reimann-Philipp et al., 1989).

We report here on the processing of type-II thionins in barley leaves by a new vacuolar proteinase which differs from previously reported ones, according to the following criteria: (a) it is not inhibited by any of the diagnostic inhibitors, including pepstatin A, which is specific for aspartic proteinases, and inhibitors of cysteine proteinases; (b) substrate cleavage specificity; (c) partial amino acid sequence information; and (d) apparent molecular mass.

MATERIALS AND METHODS

Biological material. *Hordeum vulgare* L. (cv. Bomi) seeds were grown in sterile conditions in the dark and 5–7-day-old leaves were used as experimental material. Barley leaf thionin DG3 was a gift from Dr A. Molina (Molina et al., 1993) and the acidic peptide expressed in *Escherichia coli* was donated by Dr A. Castagnaro (Madrid).

Antibodies and immunochemical methods. A fusion protein comprising the maltose binding protein of *E. coli* and the precursor of barley leaf thionin DG3 (Bohlman and Apel, 1987) was used to produce anti-precursor antibodies (Fig. 1). A *Hin*dII–*Hin*dIII DNA fragment coding for the fusion protein was ligated into the *Asp*700 (*Xmn*I) and *Hin*dIII sites of the pMAL-p2 plasmid (New England BioLabs). The fusion protein (53.3 kDa) was expressed in *E. coli* cells and purified using the protein fusion and purification system according to the manufacturer's directions (New England BioLabs). In some experiments, the fusion proteins were radiolabeled *in vivo* as described (Sambrook et al., 1989).

To obtain polyclonal antibodies against the fusion protein, doses of 100 µg were injected subcutaneously into adult female rabbits, together with Freund's complete adjuvant (Difco) at 3–4-week intervals (Tai and Chey, 1978). Proteins separated by SDS/PAGE were electroblotted onto Immobilon-P (Millipore). The immunoblotting reaction was performed using each solution of the antibody against the above fusion protein diluted 1:100 followed by incubation in goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) diluted 1:5000. Proteins were immunoprecipitated from ^{35}S-labeled extracts by incubation with antibodies against the thionin precursor or against lipid transfer protein (Molina and García-Olmedo, 1993), which had been previously bound to protein-A–agarose (Boehringer Mannheim). Anti-(thionin precursor) (dilution 1:10) or anti-(lipid transfer protein) (1:100) antibodies were incubated with swollen protein-A–agarose beads in Tris-Nonidet/NaCl (20 mM Tris/HCl pH 8.2, 150 mM NaCl, 0.5% Nonidet P-40, and 0.25 mg/ml BSA) at room temperature for 2 h with continuous shaking. Protein extracts were diluted twofold with Tris/Nonidet/NaCl and added to the antibody complexes and the mixtures were further mixed by tumbling for 4–16 h at 4°C. The protein-A–agarose beads were washed six times with Tris/Nonidet/NaCl and twice with Tris-buffered saline (20 mM Tris/Hcl pH 8.2, 150 mM NaCl), before releasing the bound proteins by incubation at 100°C for 3 min in SDS sample buffer (Laemmli, 1970). SDS/PAGE was performed as described by Laemmli (1970) using 4–20% acrylamide gradient gels (BioRad). Gels containing radiolabeled proteins were treated with Enlightning (DuPont) prior to fluorography.

Tissue labeling and fractionation. Young etiolated barley leaves were cut into 8-mm-long fragments and floated onto 40-µl drops of water (five fragments/drop), containing 80 µCi $^{35}SO_4^{2-}$ (10–1000 Ci/mol; DuPont) for the time periods indicated, followed by incubation in 40-µl drops of water. Inhibitors were added at the following concentrations: 10 µM monensin (Sigma), 50 µg/ml brefeldin A (Sigma), 10 mM zinc acetate, or 10 mM copper acetate. Thionin and its precursor were extracted with 0.5 M NaCl after freezing the leaves in liquid N_2 and grinding to a fine powder with a mortar and pestle. Insoluble material was removed by centrifugation at 10000 *g* for 15 min at 4°C and the supernatant was used for immunoprecipitation.

The microsomal fraction was obtained from labeled leaves which were homogenized as above in a buffer consisting of 12% sucrose, 250 mM Tris pH 8.5, 25 mM EDTA, 5 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$). The homogenate was centrifuged at 10000 *g* for 10 min at 4°C. The supernatant was filtered through miracloth and centrifuged at 100000 *g* for 2 h at 4°C. The microsomal pellet was suspended in Tris/Nonidet/NaCl buffer. Cell wall preparations were obtained as described by Giordani and Lafon (1993). Labeled fragments of barley leaves were infiltrated and the intercellular fluid was separated from leaves as described previously (Molina and García-Olmedo, 1993).

Preparation and fractionation of vacuoles. Protoplasts were isolated from young etiolated barley leaves using the procedure described by Díaz (1994). Protoplasts were resuspended in 3 ml warm (42°C) lysis medium (2.5% Ficoll-400, 0.5 M sucrose, 15 mM sodium phosphate pH 7.6, 2 mM EDTA), by pipeting the suspension up and down four or five times with a long Pasteur pipet; 7 µl 10 mg/ml neutral red was added to visualize vacuoles. When release of vacuoles was complete (≈5 min), the mixture was overlaid with 3 ml 0.3 M sucrose, 0.3 M sorbitol, 15 mM sodium phosphate pH 7.6, 2 mM EDTA and 0.5 ml 0.6 M sorbitol, 20 mM sodium acetate pH 5.5, 2 mM EDTA. The gradient was centrifuged at 200 *g* for 2 min and at 1000*g* for 4 min at 10°C. Purified vacuoles were collected from the top layer. The purity of vacuole preparations was checked by optical microscopy and by assay of the following enzymes: α-mannosidase (vacuolar marker; Van der Wilden et al., 1980), antimycin-insensitive NADH:cytochrome *c* reductase (endoplasmic reticulum marker; Shimomura et al., 1988), and inositol diphosphatase (Golgi marker; Chrispeels, 1983). Tonoplast and vacuolar sap proteins were separated by ultracentrifugation, as described by Höfte et al. (1991). When necessary, vacuole extracts were concentrated by centrifugation in a Microsep centrifugal concentrator 3 K (Filtron).

Assay of processing activity. A typical reaction mixture contained enzyme extract or purified proteinase and fusion protein (labeled or not) in 20 mM sodium acetate pH 5.5 and 10 mM dithiothreitol was incubated at 30°C for 3 h. The mixture was lyophilized and resuspended in SDS sample buffer before being subjected to SDS/PAGE. The following compounds were tested for inhibition of the proteolytic processing activity: 1 mM copper acetate, zinc acetate, mercury acetate, $PhMeSO_2F$, E64, *p*-chloromercuribenzoate, iodoacetic acid or *N*-ethylmaleimide, 0.3 mM pepstatin A or leupeptin, 5 or 10 mM EDTA, 1 or 10 mM *o*-phenanthroline, 10 µM monensin, 50 µg/ml brefeldin A. The assay mixture was incubated with the inhibitor for 20 min at 30°C before the addition of the substrate to start the reaction.

Purification of the processing proteinase. Young etiolated leaves from barley (5 g) were frozen in liquid N_2 and ground to a fine powder. Proteins were extracted in 20 ml 20 mM sodium acetate pH 5.5. Insoluble material was removed by centrifugation at 10000 *g* for 15 min. The supernatant was concentrated 20-fold by lyophilization, centrifuged as above and filtered through 0.45-µm filter (Schleicher & Schüll). The filtrate (1 ml) was loaded onto a Superdex 75 HR 10/30 HPLC column (Pharmacia LKB) at a flow rate of 0.3 ml/min, using 20 mM sodium acetate pH 5.5 as eluent, and 0.6-ml fractions were collected. Processing activity was assayed in all protein-containing fractions and the active fractions were used for further purification by chromatography on a Mono S HR 5/5 column at 0.5 ml/min flow rate in 20 mM sodium acetate pH 5.5. A step gradient (0–30%, min 6–16, and 30–100%, min 16–26, of 0.5 M NaCl) was used to elute proteins, and 0.5-ml fractions were collected. Active fraction (II) was concentrated by lyophilization and pooled together with other identically purified fractions. These active fractions were loaded onto a Superdex 75 HR 10/30 column at a flow rate of 0.3 ml/min and 0.6-ml fractions were collected. Processing activity was assayed in all protein-containing fractions.

Samples from the different purification steps were analyzed by 12% SDS/PAGE and identified by Coomassie or silver staining. Pre-stained molecular markers (Amersham) used for apparent molecular mass determination were: myosin (213 kDa), β-galactosidase (123 kDa), bovine serum albumin (85 kDa), ovalbumin (50.3 kDa), carbonic anhydrase (33.3 kDa), soybean trypsin inhibitor (28.5 kDa), and lysozyme (18.9 kDa). Protein concentration was estimated by the Bradford dye binding assay (Bradford, 1976), using BSA as standard; the amino acids were sequenced by automated Edman degradation.

Site-directed mutagenesis. The processing site between the mature thionin and the acidic peptide of the fusion protein was altered using the altered sites *in vitro* mutagenesis system kit (Promega) and cloning of the fusion gene in the *Sac*I and *Hin*dIII sites of the pSELECT plasmid. The mutagenic synthetic oligonucleotides used were 5′-CCTAGGGACTGGAATCACCTTAATCTTC, 5′-GGGACTATGCTAACCTTAATCT, 5′-CTATCCTAACGATAATCTTC, 5′-CTATCCTAGGCTTAATA, and 5′-GACTATCCTGACCTTAATC.

RESULTS

***In vivo* processing of thionin precursors.** A fusion protein was obtained in *E. coli* that consisted of the MalE protein, the mature leaf thionin DG3 (Bohlman and Apel, 1987), and the corresponding acidic peptide, using the pMAL-p2 plasmid (Fig. 1 A, B). Rabbit antibodies raised against the fusion protein recognized both the mature thionin (5 kDa) isolated from barley leaves and the acidic peptide produced in *E. coli* (Fig. 1 C). The faint band that appears in lane TH (at about 10 kDa) corresponds to a small proportion of thionin dimers that is usually observed when purified, unblocked protein is subjected to SDS/PAGE.

To investigate the possible accumulation of the acidic C-terminal peptide and to confirm previous reports concerning the subcellular location(s) of type-II thionins in barley leaves, intact leaf sections were washed after a 8-h radioactive pulse plus a 24-h incubation in water without label, under conditions in which cell-wall-associated lipid transfer proteins were extracted (Molina and García-Olmedo, 1993). Labeled thionins were detected by immunoprecipitation in the residual fraction, but not in the extract, while the acidic peptide was not detected in either fraction (Fig. 2 A). Similarly labeled leaf sections were homogenized and fractionated into a crude cell-wall preparation and a

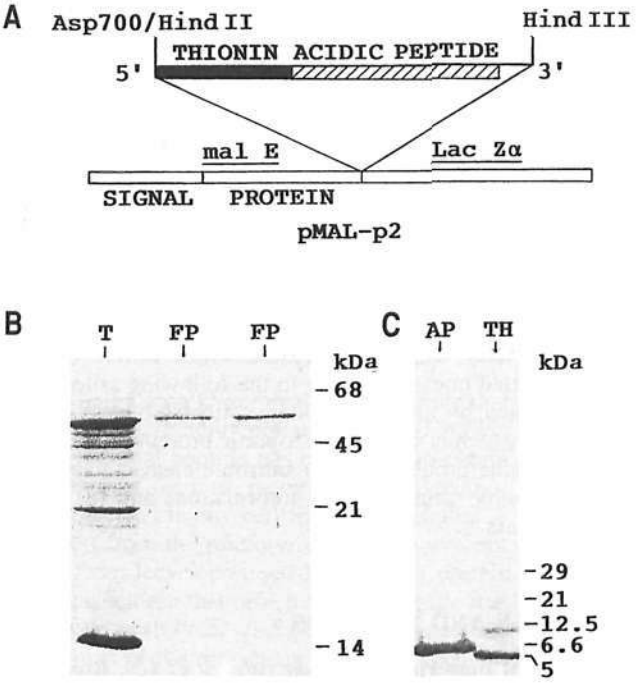

Fig. 1. Fusion protein expression and immunodetection of thionin precursor domains. (A) Schematic representation of the thionin precursor contruct in the pMAL-p2 plasmid. The signal peptide (SIGNAL) and mature protein (PROTEIN) regions of the *malE* gene are indicated. (B) Coomassie-blue-stained gel of total periplasmic fraction (lane T) of *E. coli* expressing the fusion protein and purified fusion protein (lanes FP). (C) Western blot analysis of the acidic peptide (AP) produced in *E. coli* and of purified thionin DG3 from young barley leaves (TH) with an antiserum against the MalE-thionin-precursor fusion protein.

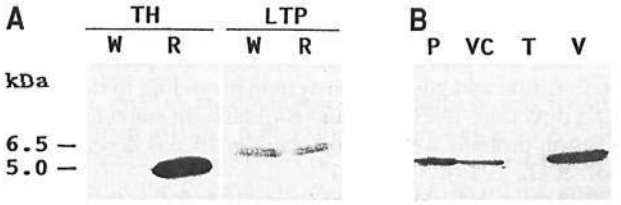

Fig. 2. Vacuolar location of type-II thionin in barley leaves. (A) Fluorograph of high-salt washed fraction (W) and the residue (R) immunoprecipitated with antibodies against MalE-thionin-precursor fusion protein (TH) or barley lipid transfer protein (LTP). Young etiolated barley leaves were pulsed with $^{35}SO_4^{2-}$ for 8 h and incubated in water without label for 24 h before fractionation. (B) Western blot analysis of protoplasts (P), vacuoles (V), vacuolar content (VC) and tonoplast (T) of barley leaves with the antibodies against the thionin precursor. Essentially the same amounts relative to α-mannosidase were loaded into the gel in lanes P and V.

supernatant fraction; less than 1% of the total of radioactive thionin was detected in the cell-wall fraction and again no trace of the acidic peptide was found (data not shown). Intact vacuoles were obtained from barley leaf protoplasts and fractionated into tonoplast and vacuolar content. Western blot analysis indicated that thionins were present only in the vacuolar content (Fig. 2 B), and thionin yields [expressed relative to the vacuolar marker α-mannosidase (Van der Wilden et al., 1980) loaded onto the gel] were essentially the same for the protoplast and the vacuole samples. The lack of detection of the excised, acidic C-terminal peptide suggested its degradation *in vivo*.

Processing of the thionin precursor into the mature protein was observed by *in vivo* radioactive labeling of tissue sections

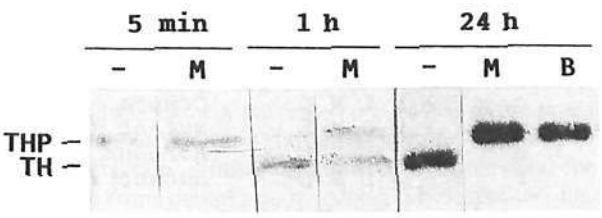

Fig. 3. *In vivo* processing of thionin precursor and inhibition by monensin and brefeldin A. Fluorograph of extracts from barley leaves labeled for 3 h with $^{35}SO_4^{2-}$ in the absence (−) or presence of inhibitor (M, 10 µM monensin; B, 50 µg/ml brefeldin A) and then incubated without label but with inhibitor for the times indicated. Antibodies against the fusion protein were used. Positions of thionin precursor (THP) and mature thionin (TH) are indicated.

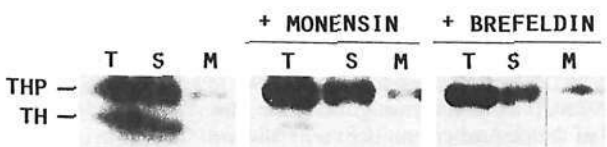

Fig. 4. Effect of monensin and brefeldin A on transport and processing of thionin precursors. Fluorograph after a 5-h pulse plus 1-h incubation in water. Fractionation into supernatant (S) and microsomal fraction (M). T, total leaf extract. Fractions were immunoprecipitated with antibodies against the fusion protein. Positions of thionin precursor (THP) and mature thionin (TH) are indicated. 10 mg of fresh tissue was used to prepare the total extract and the same amount of tissue was used to fractionate it into microsomes and supernatant.

(Fig. 3). Processing was inhibited by both monensin and brefeldin A (Fig. 3), toxins that are known to perturb the Golgi-based secretory machinery and transport of vacuolar proteins (Bednarek and Raikhel, 1992; Gómez and Chrispeels, 1993; Klausner et al., 1992; Mollenhauer et al., 1990; Pelham, 1991; Stinissen et al., 1985). A faint band of mature thionin was observed in the inhibited sample at 1 h (label+inhibitor, 3 h; plus inhibitor, 1 h) which was not observed at 24 h (Fig. 3), indicating some delay in the onset of inhibition and a rapid turnover of the mature protein under the experimental conditions used. Although the radioactive precursor was withdrawn after 3 h, incorporation of the $^{35}SO_4^{2-}$ label continued during the subsequent incubation, probably due to a lag in its diffusion and its incorporation into protein.

Tissue homogenates were analysed after radioactive labeling by fractionation into a microsomal and a supernatant fraction (which also included the vacuolar content), immunoprecipitation, electrophoresis and fluorography (Fig. 4). The mature thionin was not detected in the microsomal fraction of the non-inhibited sample, which indicated that processing must occur after the precursor leaves the microsomes. Retention of the thionin precursor in the microsomal fraction was not observed in the samples in which processing had been inhibited by monensin or brefeldin A, suggesting that retention in the particulate fraction was not the reason why cleavage of the precursor was prevented by these toxins (Fig. 4). Nigericin, an ionophore that is known to disturb or even re-direct the movement of Golgi vesicles (Craig and Goodchild, 1984), also inhibited processing but not transport of the thionin precursor (result not shown). The activity of the microsomal enzymes in the supernatant fraction was less than 1% of that in the microsomes, whereas less than 1% of the vacuolar marker activity was present in the microsomal fraction. A method to confirm whether prothionin is transported to the vacuole but not processed in the presence of either monensin of brefeldin A is to isolate vacuoles from protoplasts in the presence of the toxins. Unfortunately, attempts to obtain protoplasts from tissue treated with monensin or brefeldin A were not successful.

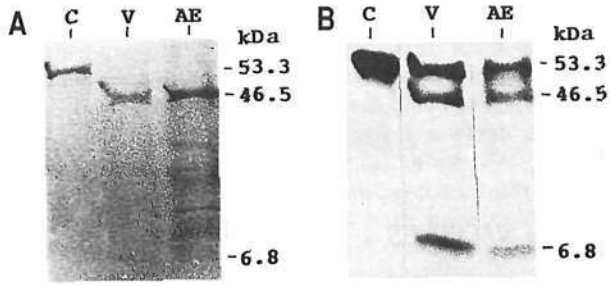

Fig. 5. *In vitro* processing of the fusion protein containing the thionin precursor. (A) Fractionation by SDS/PAGE of reaction products of the fusion protein incubated for 3 h with intact vacuoles (V) or with the acid extract from leaves (AE); (C) untreated control. Gel stained with Coomassie blue. (B) Fluorograph of the same gel.

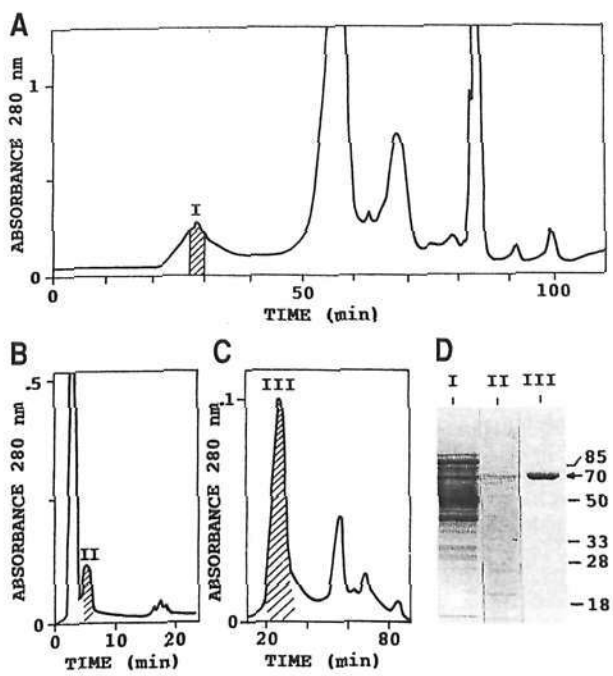

Fig. 6. Purification of the proteinase. (A) Gel filtration chromatography of the pH-5.5 extract from barley leaves on a Superdex 75 HR HPLC column (see Materials and Methods). (B) Chromatography of fraction I from A on a Mono S HR column. (C) Gel filtration on Superdex 75 HR of fraction II from B. (D) SDS/PAGE of fractions I, II, and III from A, B and C. Gels were stained with Coomasie blue. Active fractions in each step are shaded.

Characterization of the thionin precursor processing activity. To characterize the activity responsible for the cleavage of the thionin precursor, the fusion protein, which includes the complete precursor sequence, was used as a substrate *in vitro*. This protein was radioactively labeled and incubated either with intact vacuoles or with an acid (pH 5.5) protein extract from barley leaves, and the incubation mixtures were fractionated by SDS/PAGE, followed by Coomassie blue staining (Fig. 5A) or fluorography (Fig. 5B). Both intact vacuoles and the leaf extract cleaved the fusion protein (53.3 kDa) into a fragment of 46.5 kDa, that would correspond to the MalE protein plus the mature thionin and a fragment of 6.8 kDa, that would represent the acidic C-terminal peptide (Fig. 5B). Longer incubations led

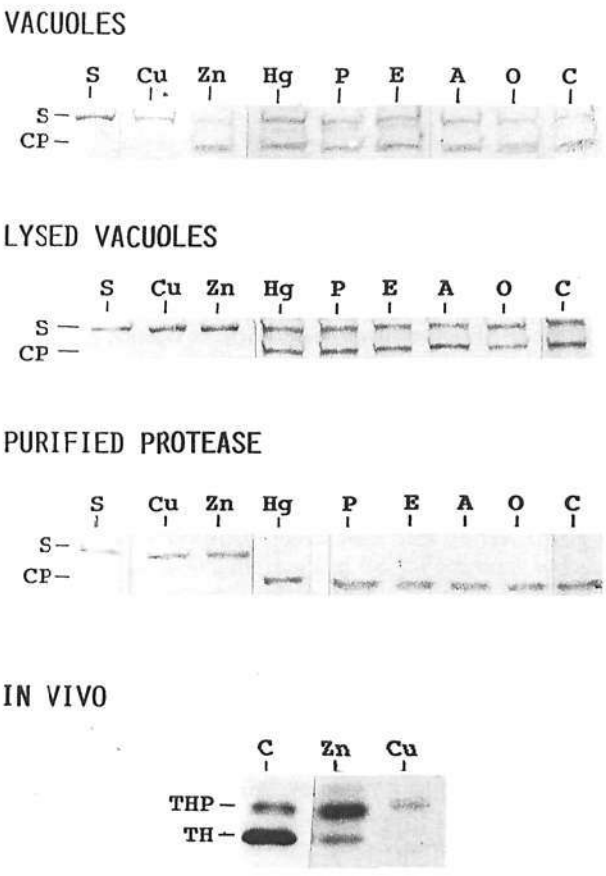

Fig. 7. Inhibition of processing activity. Fusion protein incubated with intact vacuoles, lysed vacuoles or the purified proteinase, without any inhibitor (C) or with the following proteinase inhibitors: 1 mM copper acetate (Cu); 1 mM zinc acetate (Zn); 1 mM mercury acetate (Hg); 1 mM $PhMeSO_2F$ (P); 1 mM E64 (E); 0.3 mM pepstatin A (A); and 10 mM *o*-phenanthroline (O). Substrate alone without processing extract (S). Reaction mixtures were separated by SDS/PAGE and the gels stained with Coomassie blue. Positions of the substrate (S) and of the cleaved MalE-thionin peptide (CP) are indicated. Vacuoles were lysed by vortexing. In the final gel (IN VIVO), leaf tissue was labeled for 5 h without inhibitor (C) or in the presence of 10 mM zinc acetate (Zn) or of 10 mM copper acetate (Cu). Extracts were immunoprecipitated with the antibodies against the fusion protein. Precipitated products were separated by SDS/PAGE and detected by fluorography. Positions of thionin precursor (THP) and mature thionin (TH) are indicated.

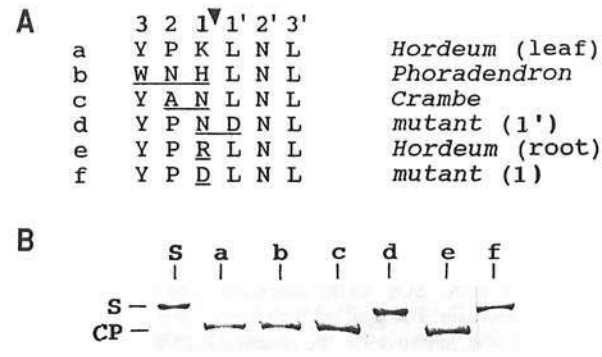

Fig. 8. Processing of fusion proteins with mutant cleavage site sequences. (A) Amino acid sequences near the cleavage site (arrowhead) of the thionin DG3 precursor (a) and of the indicated mutants (b–f) created in the fusion protein by site-directed mutagenesis as described in Materials and Methods. Altered residues are underlined. (B) Fusion proteins listed in A were incubated with the proteinase (a–f); untreated thionin DG3 fusion protein (S). Reaction mixtures were separated by SDS/PAGE and stained with Coomassie blue. Positions of substrate (S) and of the cleaved protein (CP) are indicated.

to the disappearance of the latter, but not of the 46.5-kDa fragment (not shown). The acidic peptide fragment stained poorly with Coomassie blue, which is an acidic stain, and seemed to interfere with the staining capacity of the fusion protein as compared with MalE-thionin (Fig. 5A), whereas the six Cys and three Met residues in the acidic peptide greatly enhanced the radioactive labeling and detection of the fusion protein (Fig. 5B). The processing activity required the presence of at least 2 mM dithiothreitol in the assay buffer and was optimal at 50 mM dithiothreitol.

Purification of the processing proteinase. The acid protein extract from barley leaves was fractionated on a Superdex 75 column (Fig. 6A) and the fraction I that was able to process the fusion protein was chromatographed on a Mono S column to obtain active fraction II (Fig. 6B). Chromatography of fraction II on Superdex 75 allowed the isolation of an active protein that was homogeneous as judged by SDS/PAGE, and had an apparent molecular mass of 70 kDa (Fig. 6D, III). The purified protein used for sequence analysis appeared to be blocked at the N terminus, but an 18-residue internal amino acid sequence was obtained and it was used to search for sequence similarity in the EMBL database. No significant evidence of similarity of this sequence with respect to previously described sequences was found using the FASTA, tFASTA, BLAST, and tBLAST algorithms.

Inhibition profile of the proteinase. To investigate the possible mechanism of the processing activity, as well as its possible relationship to that of previously reported proteinases, its susceptibility to a wide range of inhibitors was tested. *In vitro* inhibition was studied in parallel with intact vacuoles, lysed vacuoles, and the purified proteinase, using the fusion protein as substrate. In the three cases, a clear pH optimum at about pH 5.5 was observed (activities at pH 7.0 and 4.0 were $<25\%$ of optimum; not shown). Results obtained with diagnostic inhibitors, namely $PhMeSO_2F$ (serine proteinases), E64 (cysteine proteinases), pepstatin A (aspartic proteinases) and *o*-phenanthroline (metalloproteinases), as well as with Cu^{2+}, Zn^{2+}, and Hg^{2+}, are presented in Fig. 7. Additionally, the following inhibitors were used: iodoacetate, EDTA, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, antipain, leupeptin, and aprotinin, as well as the drugs monensin and brefeldin A (not shown). Inhibition was not observed with any of the inhibitors tested, except with Cu^{2+} and Zn^{2+}, although the latter did not inhibit processing by intact vacuoles (Fig. 7). Both metal ions inhibited processing when externally applied at the same time as $^{35}SO_4^{2-}$ in an *in vivo* pulse experiment (Fig. 7).

Cleavage specificity. Cleavage specificity was studied by site-directed mutagenesis of the fusion protein used as substrate. Altered fusion proteins were obtained in which amino acid sequences around processing sites from other thionin precursors were reproduced or the polarity of the P1 (C-terminal residue of the processing site) or P1′ (N-terminal residue of the processing site) residues was changed (Fig. 8A). Mutations that represented cleavage sites from other known thionin precursors, both from barley and from distant taxa (*Loranthaceae*, *Cruciferae*), were readily processed, whereas introduction of an aspartate residue at positions P1 or P1′ prevented processing (Fig. 8B).

DISCUSSION

The above results indicate that thionin precursors are processed in vacuoles and that the mature protein is accumulated

in the vacuolar content, while the acidic C-terminal peptide is degraded *in vivo*. The function of this peptide has not been yet investigated, although it could be involved in vacuolar targeting. Monensin and brefeldin A prevented processing of the precursor *in vivo* but not *in vitro* and did not determine retention in the microsomal fraction. Similar results have been obtained for the 11S globulin from pumpkin cotyledons, whose processing, but not targeting, is inhibited by monensin *in vivo*, but not *in vitro* (Hayashi et al., 1988). The pH optimum of the vacuolar processing activity is about pH 5.0 and inhibition *in vivo* by ionophores can be ascribed to an elevation of vacuolar pH (Tartakoff, 1983). Different ionophore effects have been observed for other plant proteins, such as re-direction of transport from the vacuole to noplast to the plasmalemma (Craig and Goodchild, 1984) or blocked transport (Gómez and Chrispeels, 1993).

The inhibition profiles of lysed vacuoles and of the purified proteinase, using the fusion protein as substrate, were identical and did not correspond to any of the four classical mechanistic types of proteinases (Salvesen and Nagase, 1989) or to previously described vacuolar processing activities. The action of most diagnostic inhibitors of cysteine proteinases may be affected by dithiothreitol, which is required for enzyme activity. However, the cysteine proteinase inhibitor E64, which is not affected by dithiothreitol (see Salvesen and Nagase, 1989), did not inhibit the processing activity, which suggests that the enzyme is not a cysteine proteinase. However, a putative thiol proteinase from jack bean was only partially inhibited by 0.5 mM E64 (Abe et al., 1993), half the concentration used in this study, and a mammalian cysteine proteinase was not inhibited by 10 μM E64 (Nicholson et al., 1995), so the possibility that the proteinase reported here belongs to this class of enzymes can not be excluded.

High concentrations of Zn^{2+} (in the millimolar range) have been shown to inhibit metallo-proteinases due to the formation of zinc monohydroxide that bridges the catalytic zinc ion to a side chain in the active site, whereas non-competitive inhibition by other heavy metals, including Cu^{2+}, is attributed to binding to other sites (Mallya and van Wart, 1988). However, activity of the proteinase, though inhibited by Zn^{2+} and Cu^{2+}, was not affected by chelators, such as *o*-phenanthroline or EDTA, so it is not possible to conclude that it corresponds to a typical metalloproteinase. It has been shown that the proglobulin processing enzyme in vacuoles from developing pumpkin cotyledons is inhibited in the presence of thiol reagents, such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetic acid, Hg^{2+}, and Cu^{2+}, and not inhibited by a number of other inhibitors, including 1 mM Zn^{2+}, which only inhibited about 20% of the activity (Hara-Nishimura and Nishimura, 1987). In contrast, the activity reported here was inhibited by Cu^{2+} and Zn^{2+} and not by any of the other inhibitors, including Hg^{2+}. Pepstatin A, which is known to inhibit processing of 2S albumin precursors from *Arabidopsis* (D'Hondt et al., 1993) and, what is more relevant, of probarley lectin from leaf vacuoles (Runeberg-Roos et al., 1994), had no effect on processing of thionin precursor. *In vitro* activity of intact vacuoles is not inhibited by Zn^{2+} probably because either a pH gradient across the tonoplast, or vesicle-mediated transport to the vacuoles, is required for inhibitory concentrations of this ion to build up inside the vacuole. The observation of cleavage inhibition by Zn^{2+} and Cu^{2+} in the intact tissue indicates that the activity characterized *in vitro* operates *in vivo* to process thionin precursors.

The cleavage site of the thionin precursor is fairly conserved at the P1 residue, which is usually Lys or His and, less frequently, Asp or Arg (see García-Olmedo et al., 1989). Site-directed mutagenesis around the processing site to reproduce the sequences corresponding to precursors of different thionins gave substrates that were readily processed *in vitro*, whereas mutations changing the polarity of the P1 or P1′ residues (Lys into Asp or Leu into Asp) led to substrates that were not processed. The latter observation is particularly relevant because processing cysteine proteinases previously described in plants are able to process Asn-Asp sites (Hara-Nishimura et al., 1991, 1993). A wide range of precursors of different proteins from seeds and mature leaves from various species have been reported to be processed by a single vacuolar thiol proteinase activity from either castor bean or pumpkin (Hara-Nishimura et al., 1991, 1993). In all these cases, Asn was invariably the P1 residue, while the P1′ residue was either neutral (Gly, Ala, Leu or Pro), acidic (Asp or Glu), or basic (Lys or Arg). Proglycinin is also processed at an Asn-Gly (P1-P1′) site and seems to require a glutamate at the P4′ position (Scott et al., 1992), whereas a conserved glutamate is not found in the known thionin precursors (Bohlmann and Apel, 1987; Gausing, 1987; Hernández-Lucas et al., 1986; Ponz et al., 1986; Schrader and Apel, 1991). Cleavage specifity of the barley proteinase reported here is also incompatible with those found for the aspartic proteinases from *Arabidopsis* (D'Hondt et al., 1993) and barley (Kervinen et al., 1993).

The apparent molecular mass of 70 kDa estimated for the processing proteinase reported here is much higher than those of cysteine proteinases from castor bean (Hara-Nishimura et al., 1993) and jack bean (Abe et al., 1993) and lower than that assigned to the proglycinin-processing enzyme (Scott et al., 1992).

In conclusion, susceptibility to inhibitors, processing-site specificity, partial amino acid sequence, and apparent molecular mass of the vacuolar proteinase described here differ from those of enzymes previously reported as responsible for cleavage of other vacuolar protein precursors from plants. The fact that processing sites of thionin precursors from wide-ranging species are cleaved suggests that this activity is probably widely distributed in the plant kingdom.

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