Identification of a Membrane-associated Cysteine Protease with Possible Dual Roles in the Endoplasmic Reticulum and Protein Storage Vacuole*

SH-EP is a vacuolar cysteine proteinase from germinated seeds of Vigna mungo. The enzyme has a C-terminal propeptide of 1 kDa that contains an endoplasmic reticulum (ER) retention signal, KDEL. The KDEL-tail has been suggested to function to store SH-EP as a transientzymogen in the lumen of the ER, and the C-terminal propeptide was thought to be removed within the ER or immediately after exit from the ER. In the present study, a protease that may be involved in the post-translational processing of the C-terminal propeptide of SH-EP was isolated from the microsomes of cotyledons of V. mungo seedlings. cDNA sequence for the protease indicated that the enzyme is a member of the papain superfamily. Immunocytochemistry and subcellular fractionation of cotyledon cells suggested that the protease was localized in both the ER and protein storage vacuoles as enzymatically active mature form. In addition, protein fractionations of the cotyledonary microsomes and Sf9 cells expressing the recombinant protease indicated that the enzyme associates with the microsomal membrane on the luminal side. The protease was named membrane-associated cysteine protease, MCP. The possibility that a papain-type enzyme, MCP, exists as mature enzyme in both ER and protein storage vacuoles will be discussed.

The endoplasmic reticulum (ER) is the port of entry of proteins into the endomembrane system. In this organelle, there are a number of soluble proteins, membrane proteins, and molecular chaperones which are involved in folding, glycosylation, assembly, and maturation of nascent proteins (1–3). The soluble proteins localized in the lumen of the ER have a retention signal, KDEL or HDEL, at the C terminus (4–6), and this signal is known to be recognized by the K(H)DEL receptor on the Golgi complex, which mediates retrieving K(H)DEL-tailed proteins to the ER. The molecular mechanisms of the ER retention of soluble proteins are conserved through animal, plant, and yeast cells (7–9).

In several kinds of plants, unique papain-type proteinases possessing a C-terminal KDEL sequence have been identified (10–16). One such KDEL-tailed cysteine protease, designated SH-EP, was first isolated from cotyledons of germinated Vigna mungo seeds as the enzyme responsible for degradation of storage proteins accumulated in protein storage vacuoles (PSV) of cotyledon cells (10, 17). SH-EP is synthesized on membrane-bound ribosomes as a 43-kDa precursor through co-translational cleavage of the signal peptide, and the precursor is processed to the 33-kDa mature enzyme via 39- and 36-kDa intermediates during or after transport to the vacuoles (18, 19).

The function of the KDEL-tail on a cysteine protease whose final destination is the PSV has been an interesting question. Based on analysis of the heterologous expression of SH-EP and a KDEL deletion mutant of SH-EP in insect Sf9 cells and subcellular fractionation of cotyledon cells, it was proposed that the KDEL-tail of SH-EP functions to store the enzyme as a transientzymogen in the ER, and that the conversion of the 43-kDa SH-EP into the 42-kDa form in/at the ER is accompanied by the removal of the C-terminal propeptide containing the KDEL-tail (20). From these observations, the protease responsible for removal of the C-terminal propeptide of SH-EP has been supposed to exist in the lumen of the ER (20). Recently, Toyooka et al. (21) showed that in cotyledon cells of V. mungo seedlings, a proform of SH-EP synthesized in the ER accumulated at the edge or middle regions of the ER where the transport vesicle was formed. The vesicle, containing a large amount of pro-SH-EP, termed KV, budded off from the ER, bypassed the Golgi complex, and fused to PSV (21). It was proposed that the KDEL-tail of SH-EP functions as the signal for accumulation of pro-SH-EP at the edge or middle regions of the ER where formation of KV proceeds (21).

In this study, the protease involved in the processing of 43-kDa SH-EP (KDEL-attached form) into 42-kDa SH-EP (KDEL-removed form) was purified to homogeneity from microsomes of cotyledons of V. mungo seedlings, and a cDNA clone for the enzyme was isolated. Immunocytochemical and sucrose gradient analyses of cotyledon cells were carried out to determine the intracellular localization of the protease. The protease was also expressed in insect Sf9 cells in order to observe the characteristics of the membrane association of the enzyme. The possible dual functions of this enzyme in cells will be discussed.

**EXPERIMENTAL PROCEDURES**

*Plant Materials—* V. mungo seeds were germinated on layers of wet filter paper at 27 °C in darkness, and cotyledons were collected on day 3 of post-imbibition.
Membrane-associated Cysteine Protease

SDS-PAGE and Immunoblotting—SDS-PAGE was conducted on 12.5% gels, and immunoblotting was performed as described elsewhere (18).

MCP Assay—An active site mutant of 43-kDa SH-EP in which the active site was replaced by Gly was expressed in Escherichia coli BL21 (DE3), recombinant 43-kDa SH-EP (C152G) was purified to homogeneity and used as substrate to assay the enzymatic activity of MCP. Ten microliters (μL) of the substrate and 10 μL of enzyme solution were mixed and incubated at 27 °C for 16 h. After incubation, 20 μL of SDS-PAGE sample buffer (20 mM Tris-Cl, pH 6.8, 6% glycerol, 2% SDS, 0.1% bromphenol blue, 10% 2-mercaptoethanol) was added and the mixture was boiled for 5 min. The mixture was loaded onto a 12.5% SDS-PAGE (Coomassie Brilliant Blue (CBB) R staining).

Purification of MCP and Determination of Its N-terminal Amino Acid Sequence—All manipulations were conducted at 0 to 4 °C. Cotyledons (200 g) of day 3 dark-grown seedlings were homogenized with 600 mL of 0.1 M Tris-Cl (pH 7.4) containing 0.44 mM sucrose, 1 mM EDTA, and 0.1 mM MgCl₂. The homogenate was centrifuged at 800 × g for 10 min and then at 4,500 × g for 30 min. The supernatant was again centrifuged at 100,000 × g for 60 min, and the precipitate was washed with homogenization buffer and resuspended with 120 mL of buffer. The resuspended solution was sonicated (3 × 1 min, 30 W, U-200P, Tomy Seiko Co., Ltd.), and centrifuged at 100,000 × g for 60 min. The precipitate was then resuspended with 50 mM sodium phosphate buffer (pH 7.2) containing 1% n-octyl-β-D-thioglucoside and 10 mM n-octyl-β-D-thioglucoside. The resuspended solution was again centrifuged at 100,000 × g for 60 min, and the supernatant was used as the starting material for column chromatography procedures. The solution was applied to a column (1.6 × 20 cm) of DEAE-cellulose (Whatman DE-52) that had been pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.2) containing 0.1% n-octyl-β-D-thioglucoside and 10 mM n-octyl-β-D-thioglucoside. The loaded column was then washed with the same buffer and subsequently eluted with a linear gradient (100 μL/100 mL) of 0 to 0.5 M KCl in the buffer. The eluate was collected in 10-mL fractions at a flow rate of 1.5 mL/min. The active fractions (60 mL) were placed in a dialysis tube and the volume was reduced to 3 mL by use of polyethylene glycol 20,000. The concentrated solution was loaded onto a column (1.0 × 75 cm) of Sephacryl S-200 (Amersham Pharmacia Biotech) which was pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.2) containing 1% n-octyl-β-D-thioglucoside and 10 mM n-octyl-β-D-thioglucoside. The solution was then dialyzed against distilled water, and the dialyzed solution was filtered through a Sepharose 4B (Amersham Pharmacia Biotech) column that had been pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.4) containing 0.44 M sucrose, 1 mM EDTA, and 0.1 mM MgCl₂. The column was eluted with 0.1 M Tris-Cl (pH 7.4) containing 0.4 M sucrose, 1 mM EDTA, and 0.1 mM MgCl₂. The homogenate was centrifuged at 800 × g for 10 min and then at 4,500 × g for 30 min. The supernatant was again centrifuged at 100,000 × g for 60 min, and the precipitate was washed twice with the homogenization buffer and used as the microsomal fraction.

Electron Microscopy and Immunocytochemistry—Day 3 cotyledons of V. mungo seedlings were fixed in 2% formaldehyde, 2% glutaraldehyde in 50 mM potassium phosphate buffer (pH 7.4) containing 1% w/v sucrose, 10% w/v 2-mercaptoethanol, and 0.1% w/v glutaraldehyde. The tissue pieces were sonicated in a grade methanol series and embedded in a hard formulation of LR White resin. Ultrathin sections mounted on nickel grids were blocked with 5% bovine serum albumin in TBST (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.2% Tween 20) for 15 min at room temperature. The sections were then incubated with affinity-purified antibody against MCP for 30 min at room temperature. Sections were then washed with TBST and indirectly labeled with 10-nm colloidal gold anti-rabbit IgG in TBST for 15 min at room temperature. The grids were then washed with TBST followed by distilled water, and then stained with 5% uranyl acetate for 20 min. The grids were examined and photographed with a transmission electron microscope (model 1010EX, JEOL) at 80 kV. Ultrastructural analysis was carried out according to Hara-Nishimura et al. (27).

Preparation of Recombinant Baculovirus, Expression of MCP in Sf9 Cells, and Subsequent Analysis—The DNA insert of full-length MCP cDNA was cut out of pBluescript SK+ phagemid vector using NotI and subcloned into pVL1392 baculovirus transfer vector cleaved by the same enzyme. The pVL1392 vector harboring MCP cDNA and a BaculOGold™ Transfection Kit (Pharmingen), recombinant baculovirus containing MCP cDNA was prepared and amplified according to the manufacturer’s instructions. Preparation of recombinant baculovirus of SH-EP and production of MCP and SH-EP in Sf9 cells were conducted as described (20). After a 3-day infection with MCP or SH-EP baculovirus, Sf9 cells were peeled off from 25-cm² culture dishes with serum-free Grace’s medium (Life Technologies, Inc.). The resuspended cells were centrifuged at 200 × g for 5 min, and the pellet was resuspended with serum-free Grace’s medium and centrifuged again. The pellet was then resuspended with 0.1 M Tris-Cl (pH 7.4) containing 0.4 M sucrose, 1 mM EDTA, and 0.1 mM MgCl₂, and was sonicated for 1 min at 30 W. A 50-μL aliquot of the sonicated cells was retained as a whole cell extract, and the remainder of the sonicated cell preparation was centrifuged at 3,000 × g for 5 min. The supernatants were again centrifuged at 100,000 × g for 1 h. The supernatant was used as the soluble protein fraction of the cells, while the precipitate was washed with 0.1 M Tris-Cl (pH 7.4) containing 0.4 M sucrose, 1 mM EDTA, and 0.1 mM MgCl₂, solubilized with 0.5 mL of 8 M urea in 0.1 M Tris-Cl (pH 8.0) and used as the membrane protein fraction of the cells. For measurement of MCP activity of recombinant MCP, the precipitate from centrifugation at 100,000 × g was washed with 0.1 M Tris-Cl (pH 7.4) containing 0.4 M sucrose, 1 mM EDTA, and 0.1 mM MgCl₂, solubilized with 0.5 mL of 50 mM sodium phosphate buffer (pH 7.2) containing 1% Triton X-100 and 10 mM n-octyl-β-D-thioglucoside. The solubilized solution was again centrifuged at 100,000 × g for 1 h, and the supernatant was used for MCP assay.

In Vitro Processing of Recombinant 43-kDa SH-EP (C152G) by Papain—Papain (10 μg/mL, 30 milliunits/μg) was purchased from Roche Molecular Biochemicals, and the enzyme was diluted to the concentration of 10 to 1 ng/mL and 10 μL containing 0.1 M sodium phosphate buffer (pH 7.2) containing 1% Triton X-100 and 10 mM n-octyl-β-D-thioglucoside. Recombinant 43-kDa SH-EP (C152G) was prepared as above, and 10 μL (2 μg) of the substrate was mixed with 10 μL of diluted papain solution. After 12 h incubation of the mixture at 27 °C, 20 μL of SDS-PAGE sample buffer was added and the mixture was boiled for 3 min. Processed product of the substrate by papain was detected with SDS-PAGE fol.
lowed by Coomassie Brilliant Blue staining or by immunoblotting with antibody against mature SH-EP or N-terminal prosequence of SH-EP.

**RESULTS**

**Identification of Enzymatic Activity of MCP**—Recombinant 43-kDa SH-EP can be converted into the mature enzyme by autocatalysis (22). For the assay of the enzymatic activity of MCP responsible for the processing of the C-terminal proregion of SH-EP, an active site mutant, 43-kDa SH-EP (C152G), was used as a substrate to eliminate the possibility of autocatalytic processing of the substrate during incubation with the enzyme solution. The 43-kDa SH-EP (C152G) has also been shown to be correctly folded (22). When the substrate was incubated with the buffer, no degradation products of the substrate were detected (Fig. 1A, *buffer lane*), indicating that the substrate was stable and there was no contaminating protease in the preparation of the substrate. Coincubation of the substrate with the soluble protein fraction and the luminal protein fraction from microsomes showed no effect on the substrate (Fig. 1A, 100,000 × g sup and lumen lanes). The substrate was cleaved into the 42-kDa form when it was incubated with the membrane protein fraction from microsomes (Fig. 1A, *membrane lane*). The results of the MCP assay indicated that the enzymatic activity catalyzing the substrate into the 42-kDa form is localized in the membrane protein fraction of microsomes from cotyledons. This protease activity was inhibited by E-64 but not by diisopropyl fluorophosphate, pepstatin A, or EDTA (Fig. 1, B and C), suggesting that the enzyme involved in this proteolysis is a cysteine protease whose active site cysteine can be irreversibly bound to the epoxy group of E-64. In addition to the conversion of the substrate to 42-kDa form by MCP activity, the total amount of the substrate was decreased during incubation (Fig. 1, B and C). This loss of protein will be due to degradation of the substrate by proteinase(s) which coexist in the membrane protein fraction from microsomes.

To investigate whether there was C-terminal cleavage of the substrate by the MCP activity, and to determine the intracellular localization of the enzyme, the microsomes were further separated by isopycnic sucrose density gradient and subsequent analysis of each fraction from the gradient was conducted. The major enzymatic activity of MCP was detected in fractions 5 and 6 (Fig. 2A). When the reaction mixture consisting of the substrate and each fraction from the gradient was analyzed by SDS-PAGE/immunoblotting with anti-KDEL antibody, the antibody labeled only the substrate (Fig. 2B), and the density of the substrate bands decreased at fractions 5 and 6. In addition, the 42-kDa SH-EP (C152G) generated by the enzymatic activity of MCP was not recognized by the antibody. These suggest that the conversion of 43-kDa SH-EP (C152G) to the 42-kDa form by MCP activity was accompanied by the loss of the C-terminal prosequence containing the KDEL-tail. When the fractions from the sucrose gradient were analyzed by SDS-PAGE/immunoblotting with anti-BiP antisemur, an intense band of BiP was detected in fractions 5 to 7 where the MCP activity was detected (Fig. 2C), suggesting that MCP is mainly localized in the ER.

**Purification of MCP**—Using the assay method described above, MCP was purified from microsomes of day-3 cotyledons of *V. mungo* seedlings. n-Octyl-β-D-glucoside was used as a detergent during the purification procedures instead of the Triton X-100 used in the MCP assay (Fig. 1A), since Triton X-100 often interfered with the column chromatography procedures. The enzyme was purified to homogeneity by DEAE-cellulose column chromatography and gel filtration. All active fractions from the DEAE-cellulose column chromatography were applied to gel filtration (Fig. 3A, *upper panel*). The proteolytic activity to the recombinant 43-kDa SH-EP (C152G) was detected in fractions 18 and 19 (Fig. 3A). The molecular mass of MCP was 32 kDa as judged by its mobility in SDS-PAGE (Fig. 3B). The N-terminal amino acid sequence of the enzyme was determined to be Leu-Pro-Ala-Asn-Ala-Glu-Lys; EDTA, 1 mM. The homogenate was centrifuged at 500 × g for 10 min and then at 4,500 × g for 30 min. The supernatant was again centrifuged at 100,000 × g for 60 min. The supernatant was used as the 100,000 g sup. fraction, and the precipitate was washed with homogenization buffer and resuspended with 10 ml of the buffer. The resuspended solution was sonicated (3 × 1 min, 30 W) and centrifuged at 100,000 × g for 60 min. The supernatant was used as the luminal proteins of the microsomes, and the precipitate was washed with the buffer and solubilized with 50 mM sodium phosphate buffer (pH 7.2) containing 1% Triton X-100 and 10 mM 2-mercaptoethanol. The resuspended solution was again centrifuged at 100,000 × g for 60 min, and the supernatants were used as the membrane protein fraction of the microsomes. The enzymatic activity of MCP was assayed as described under “Experimental Procedures.” B, several kinds of inhibitors were added to the reaction mixture which contained the substrate and membrane protein fraction described in A. C, same reaction mixtures with B were analyzed by SDS-PAGE/immunoblotting with anti-SH-EP antibody. The concentrations of the inhibitors were: 1-trans-epoxysuccinyl-leucylamido(4-guanidinobutane) (E-64), 10 μM; diisopropyl fluorophosphate (DFP), 1 mM; pepstatin A, 10 μM; EDTA, 1 mM.

![Fig. 1. The enzymatic activity of MCP acting to process the 43-kDa SH-EP (C152G) to the 42-kDa form (A) and effects of inhibitors on the MCP activity (B).](image)

A, substrate +

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B, kDa

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C, kDa

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D, kDa

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E, kDa

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F, kDa

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G, kDa

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H, kDa

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I, kDa

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J, kDa

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K, kDa

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L, kDa

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M, kDa

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N, kDa

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Q, kDa

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W, kDa

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X, kDa

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Y, kDa

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Z, kDa

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**Cloning of MCP cDNA**—Homologous proteases with an N-terminal amino acid sequence identical to that of MCP were searched for with FASTA, and the search results indicated that MCP belongs to the papain protease family. These search results were supported by the inhibitory effect of E-64, a potent inhibitor of papain-type protease, on MCP activity. Primers
were set to the N-terminal amino acid sequences of MCP and to a conserved motif of the papain-type proteases. A DNA fragment amplified by polymerase chain reaction was used for screening of a cDNA library from day 3 cotyledons of V. mungo seedlings. The nucleotide sequence of the full-length cDNA for MCP consisted of 1414 bp (accession number AB038598), with an open reading frame of 1045 bp encoding 364 amino acid residues with a calculated molecular mass of 39491.32 Da. The deduced amino acid sequence of MCP had a putative signal sequence of 20 amino acid residues (29) and an N-terminal prosegment of 98 amino acid residues. One putative Asn-linked glycosylation site was found in the amino acid sequence of MCP. The phylogenetic tree and amino acid sequence alignment of 25 papain-type proteases are known to be destructive enzymes and to be proteases belonging to other papain subfamily.

**Intracellular Localization of MCP**—In general, the papain-type proteases are known to be degradative enzymes and to be localized in vacuoles/lysosomes or to be secreted. However, MCP was purified from the microsomal fraction and is thought to be localized in the ER. Therefore, we carried out sucrose gradient and immunocytochemical analyses of cotyledon cells to determine the intracellular localization of MCP. Each fraction of the sucrose gradients of microsomes from the cotyledons were analyzed by SDS-PAGE/immunoblotting using anti-MCP, anti-BiP, and anti-α-amylase antibodies. α-Amylase has been resolved to be localized in vacuoles of cotyledon cells of V. mungo seeds (25). The major band of MCP was detected in fractions 5–7, BiP in fractions 5–8, and α-amylase in fractions 6–9 (Fig. 5, A-C), suggesting that MCP in the microsomal fraction was localized in the ER and vacuoles. In addition, MCP was detected as an enzymatically active mature form of 32 kDa even in ER fractions (Fig. 5, A-C). Experimental details are described under “Experimental Procedures.” The results of measurements of MCP activity in separated fractions were presented in the lower panel. Inset in upper panel, MCP activity. B, fractions 16–21 in A was separated by SDS-PAGE and the gel was silver stained. Arrow indicates MCP polypeptide.

FIG. 2. Sucrose gradient centrifugation of microsomes from cotyledons of day-3 V. mungo seedlings and subsequent analysis. Microsomes prepared from 25 g of day 3 cotyledons were resuspended in 1.5 ml of buffer A (0.1 M Tris-Cl, pH 7.4, 0.44 M sucrose, 1 mM EDTA, and 0.1 mM MgCl2). The suspension was centrifuged at 200 × g for 5 min, and the supernatant was layered on a continuous 0.6–1.6 M sucrose gradient and centrifuged at 100,000 × g for 17 h. After the centrifugation was completed, the gradient was fractionated into 0.7-ml fractions. Fifty microliters of each fractionated sample was pooled for the analysis described in C. The remaining sample (0.65 ml) was mixed with 0.7 ml of buffer A, and the mixture was sonicated (1 min, 30 W). After centrifugation at 100,000 × g for 60 min, the precipitate was washed with buffer A and solubilized with 0.5 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 1% Triton X-100 and 10 mM 2-mercaptoethanol. The solution was again centrifuged at 100,000 × g for 60 min, and the supernatants were used for the procedures described in A and B. After incubation of each sample with the substrates, the mixture was separated by SDS-PAGE in duplicate. One gel was stained with Coomassie Brilliant Blue for measurement of MCP activity (A), and the other was immunoblotted with anti-BiP antibody to check for the removal of the C-terminal KDEL from the substrate (B). Each fraction from the sucrose gradient was analyzed by SDS-PAGE immunoblotting with antisera against maize BiP (C).

FIG. 3. Separation of MCP by column chromatography (A) and SDS-PAGE of purified MCP (B). A, the active fraction from DEAE-cellulose column chromatography was subjected to gel filtration on Sephacryl S-200 (upper panel). Experimental details are described under “Experimental Procedures.” The results of measurements of MCP activity in separated fractions were presented in the lower panel. Inset in upper panel, MCP activity. B, fractions 16–21 in A was separated by SDS-PAGE and the gel was silver stained. Arrow indicates MCP polypeptide.

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**FIG. 4.** Phylogenetic tree (A) and alignment of amino acid sequences (B) of papain-type proteases. 

**A.** The phylogenetic tree was constructed by the neighbor-joining method (47) without gap regions which were generated for maximum matching. Numbers along each branch are bootstrap values (48). Proteases of the MCP subfamily are bracketed. After alignment of the amino acid sequences of papain-type proteases, a portion around the N-terminal amino acid residues of the mature forms of the proteases were analyzed. The line above the sequence indicates the determined N-terminal amino acid sequence of MCP. The open arrowhead indicates the N-terminal amino acid residue of MCP. The closed arrowheads indicate N-terminal amino acid residues of papain, SH-EP, aleurain, cathespin L, cathespin B, REP-1, EPB p34, proteinase A, and oryzain α. The open circle indicates the active site cysteine. Accession numbers of sources are: MCP, AB038598; French bean CP, Z99953; soybean isoform B, U71379; pea CP, X54358; fava bean CP, U59465; vetch CPR2, Z30338; tobacco CP, Z14028; Arabidopsis RD19, D13042; soybean CP, Z32795; mouse cathespin L, P06797; Maize CP, 99936; barley aleurain, X58859; Arabidopsis RD21, D13043; rice oryzain α, D90406; barley EPB, U19339; rice REP-1, D76415; barley EPA, Z97023; rice REP-A, D76416; vetch proteinase A, Z34895; French bean EP-C1, X56753; mung bean SH-EP, X15723; soybean p34, J05560; papaya papain, M15203; mouse cathespin B, M14222.
dependent pathway (Fig. 6C). Interestingly, MCP was abundant in specific regions of PSV where degradation of storage proteins is occurring. Ultrastructural analysis of cotyledon cells indicated that degradation of storage proteins starts at the inside of PSV, and the size of the white-colored regions gradually enlarges as degradation progresses (Fig. 7C, PSV1 to PSV3). These findings strongly suggested that, in PSV, MCP plays a role in the early stage of degradation of storage proteins.

Association of MCP with Membranes—Experiments to examine the possible membrane association of MCP by ultrasonic wave treatment of microsomes from the cotyledons and subsequent ultracentrifugation were carried out since MCP activity was detected in the membrane protein fraction from microsomes (Fig. 1A). MCP was mainly detected in the membrane protein fraction of microsomes (Fig. 8A), whereas most of the SH-EP was presented in the luminal soluble protein fraction (Fig. 8B). To see whether MCP is associated with the membrane on the luminal side or the cytoplasmic side from microsomes, the microsomes of cotyledons were treated with proteinase K with or without detergent and analyzed by SDS-PAGE immunoblotting with anti-MCP antibody (Fig. 8C). MCP was intact in the absence of Triton X-100, but the addition of the detergent to the reaction mixture resulted in degradation of MCP by proteinase K, indicating that MCP associates with the microsomal membrane on the luminal side. It should be noted that SH-EP was presented as a proenzyme in the microsomes, but MCP as the mature form (Fig. 8, A and B). These suggest that MCP associates with the microsomal membrane as the mature enzyme.

Next, full-length MCP cDNA was expressed in insect Sf9 cells to see whether the association of MCP with the membrane is due to the nature of the MCP protein itself or is a localization specific for cotyledon cells. When the total proteins of Sf9 cell expressing MCP were analyzed by SDS-PAGE/immunoblotting using anti-MCP antibody, intense bands of 32.5, 35, 40, 42, and 44 kDa, and weak bands of 37 and 38 kDa were detected (Fig. 9A). The 32.5-kDa polypeptide is the putative mature MCP in Sf9 cells, and the 44-kDa polypeptide corresponds to the pro-form of MCP. It could not be determined whether the other MCP-related polypeptides were intermediates of MCP or degraded products of pro-MCP. When the extracts of the Sf9 cells were separated into luminal protein and membrane protein fractions, the 32.5-kDa MCP was detected only in the membrane protein fraction, whereas the 35-, 37-, and 38-kDa polypeptides were observed only in the luminal protein fraction. When SH-EP, a soluble enzyme, was expressed in Sf9 cells and the cells were treated as above, SH-EP-related polypeptides were detected only in the luminal protein fraction (Fig. 9B), suggesting that the separation of luminal and membrane protein fractions was successful. These expression of MCP and its subsequent specific localization in this heterologous cell system strongly suggest that the membrane association of MCP is a result of the nature of the MCP polypeptide itself.

To observe whether MCP heterologously expressed in Sf9 cells is enzymatically active, MCP activities in membrane protein fractions from Sf9 cells expressing MCP and uninfected
(wild type) Sf9 cells were measured. When the recombinant 43-kDa SH-EP (C152G) was incubated with membrane proteins from uninfected Sf9 cells, the substrate was stable (Fig. 9C). In contrast, after incubation of the substrate with membrane proteins from Sf9 cells expressing MCP, the recombinant 43-kDa SH-EP (C152G) was converted to 42-kDa form (Fig. 9C), suggesting that MCP expressed in Sf9 cells is correctly folded and activated. No MCP activity was detected in membrane proteins from uninfected Sf9 cells, however, it has been revealed that 43-kDa SH-EP expressed in Sf9 cells is cleaved to 42-kDa form by endogenous protease(s) with removal of C-terminal region (20). The possibility may be suggested that the endogenous protease in Sf9 cells which is responsible for such conversion was lost or inactivated during the preparation of membrane protein fraction from Sf9 cells, or that the enzymatic activity cannot be detected by the MCP assay system used in this study.

Processing of Recombinant 43-kDa SH-EP (C152G) by Papain—Processing pattern of the recombinant 43-kDa SH-EP (C152G) by papain was observed to see whether general cysteine protease has proteolytic activity that converts the substrate to 42-kDa form, since MCP was revealed to be a member of papain family. When the recombinant 43-kDa SH-EP (C152G) was incubated with papain, the substrate was processed to 33-kDa form. Immunoblot analysis of the processed SH-EP with antibody to mature SH-EP or to N-terminal propeptide of SH-EP indicated that the 33-kDa SH-EP cleaved by papain was not immunoreactive to the antibody specific to N-terminal prosequence of SH-EP (Fig. 10C) but to antibody against mature SH-EP (Fig. 10B). These suggest that the recombinant 43-kDa SH-EP (C152G) was processed to the 33-kDa form, which is corresponding to the mature enzyme,
insect Sf9 cells (Triton X-100 and 10 mM 2-mercaptoethanol) were mixed and incubated.

antibody (each) were analyzed by SDS-PAGE/immunoblotting with anti-MCP antibody.

active mature SH-EP (22).

(C152G) was intermolecularly processed to the mature form by consistent with the report that the recombinant 43-kDa SH-EP through intermolecular proteolysis by papain. This result is presented as the processed SH-EP and papain, respectively.

DISCUSSION

Although MCP was initially isolated as a protease which is involved in post-translational cleavage of the C-terminal propeptide of SH-EP containing the KDEL-tail within the ER, and the amino acid sequence of MCP deduced from its cDNA indicated that the enzyme is a member of papain family which are potentially degradative enzymes. The involvement of MCP in degradation of storage proteins is consistent with the report that an MCP homologue from Vicia faba was isolated as an enzyme responsible for globulin hydrolysis in cotyledons of germinated V. faba seeds (30, 31). It has been shown that there are some kinds of proteinases in the cotyledons of germinated V. mungo seeds (17, 28) and other papain-type proteinases are thought to exist in the cotyledons, however, only MCP showed the proteolytic activity that converts the recombinant 43-kDa SH-EP (C152G) to the 42-kDa form. In addition, general cysteine proteinase, papain, cleaved the recombinant 43-kDa SH-EP (C152G) to the 33-kDa form through removal of the N-terminal prosequence of the proenzyme. This processing profile is apparently different from that of the recombinant 43-kDa SH-EP (C152G) by MCP. This suggest the possibility that the processing activity for the conversion of the recombinant 43-kDa SH-EP (C152G) to the 42-kDa form is unique to MCP.

The present results of sucrose gradient centrifugation and immunocytochemistry of cotyledon cells suggest the possibility that MCP exists as an active mature enzyme in the ER in association with the membrane, although papain-type proteases have not been expected to be localized in the ER lumen since the ER is a site for folding and oligomerization of nascent proteins (1–3). However, MCP was detected as the mature enzyme in a microsome preparation in which SH-EP was presented as the proenzyme (Fig. 8, A and B), suggesting the enrichment of the ER in the microsomal preparation, and supporting the possibility that mature MCP is localized in the ER. Most papain-type proteases are synthesized as zymogens with the large N-terminal proregion which is essential for correct folding of the enzyme (32), contains intracellular transport signal (33), and plays a role in inactivation of the protein (34–36). The activation mechanisms of the proform of papain-type proteases have been elucidated mainly for propapain (32, 35), procathepsin L (37, 38), and procathepsin B (39), and the mechanisms are largely conserved among these proenzymes. The N-terminal proregion occluding the active site with anti-parallel peptide chains is removed by autocatalytic proteolysis triggered by acidification (40, 41). We have also succeeded in in vitro activation of recombinant pro-SH-EP (22), but recombinant pro-MCP was not converted to the mature enzyme under the conditions of acidic and neutral pH (data not shown). An activation mechanism different from that of pro-SH-EP, procathepsin L, and propapain may account for the activation of pro-MCP. In fact, the in vitro activation mechanism of proforms of proteases belonging to the same subfamily as MCP has not yet been resolved. Therefore, the processing profiles of MCP was analyzed by heterologous expression system in insect Sf9 cells. It is notable that mature MCP was detected only in the membrane protein fraction prepared from Sf9 cells expressing MCP, and that the other MCP-related polypeptides of 35, 37, and 38 kDa were detected only in the soluble protein fraction (Fig. 9A). In addition, MCP activity derived from expressed MCP was detected in the membrane protein fraction (Fig. 9C).
This suggests that the membrane association of pro-MCP in Sf9 cells is needed for correct processing to the mature form, and pro-MCP in the soluble protein fraction is processed abnormally or degraded to the 35-, 37-, and 38-kDa polypeptides. Membrane association may have a crucial role in post-translational processing of pro-MCP.

A hydropathy plot for the MCP protein did not show the existence of a hydrophilic transmembrane domain on the MCP polypeptide (data not shown). Membrane association of papain-type proteases has been reported in procathepsin L (42, 43) and polypeptide (data not shown). Membrane association of papain-type proteases has been reported in procathepsin L (42, 43) and polypeptide (data not shown). Membrane association may have a crucial role in post-translational processing of pro-MCP.

The results of immunocytochemistry showing that MCP is localized in both the ER and PSV as a membrane-associated protein is localized in both the ER and PSV. The microsomal membrane on the cytoplasmic side during prep-
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