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# Posttranslational processing of a carboxy-terminal propeptide containing a KDEL sequence of plant vacuolar cysteine endopeptidase (SH-EP)

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**Abstract**

A plant cysteine endopeptidase, designated SH-EP, is a major protease occurring in cotyledons of *Vigna mungo* seedlings, and acts to degrade seed globulin stored in protein bodies. Here we show that the 43 kDa intermediate of SH-EP formed in the endoplasmic reticulum is transported to protein bodies and processed to the 33 kDa mature form during transport or thereafter, and that the COOH-terminal propeptide of 10 amino acid residues containing a KDEL sequence, which is known as a retention signal for the endoplasmic reticulum lumen, is processed to form the mature SH-EP.

*Key words:* Cysteine endopeptidase; Posttranslational processing; Protein body; KDEL sequence; COOH-terminal propeptide

## 1. Introduction

SH-EP, a plant cysteine endopeptidase, was purified as one of the major proteases expressed in cotyledons of *Vigna mungo* seedlings [11]. The endopeptidase acts in vitro to degrade seed storage globulin in combination with a serine endopeptidase [10]. Analysis of the products of translation in vitro and the results of in vitro processing experiments with enzyme extracts have suggested that SH-EP of 33 kDa is synthesized on membrane-bound polysomes as a large, inactive 45 kDa precursor, which is cotranslationally processed to a 43 kDa intermediate through cleavage of a signal peptide, and that this intermediate is processed further to the 33 kDa mature enzyme through 39 kDa and 36 kDa intermediates [11]. We isolated a cDNA clone for SH-EP from a cDNA expression library of the cotyledons and determined the sequence of its insert [2]. The cloning and sequencing of the gene for SH-EP have been reported elsewhere [1]. The amino acid sequence of SH-EP deduced from the nucleotide sequence of the cDNA for SH-EP contains a tetrapeptide Lys-Asp-Glu-Leu (KDEL) at its COOH-terminus. This tetrapeptide has been shown to function as a retention signal for the ER lumen in mammalian and plant cells [3,14]. This is apparently inconsistent with the above postulation that SH-EP has a role in the degradation of storage globulin in protein bodies, or storage protein vacuoles, which have become filled with storage protein during seed maturation. We therefore attempted to determine whether or not the mature form of SH-EP has the COOH-terminal KDEL tail, and found that the mature form lacks the COOH-terminal 10 amino acid residues as compared to the sequence deduced from the nucleotide sequence of the cDNA. To the best of our knowledge, this report is the first to describe posttranslational processing of COOH-terminal propeptide containing the KDEL tail.

## 2. Materials and methods

### 2.1. Plant materials

*Vigna mungo* seeds were germinated on layers of wet filter paper at 27°C in the dark. Cotyledons were harvested at a stage of day 3 where the endopeptidase activity as well as the content of SH-EP mRNA reached maximum levels [11,16].

### 2.2. Purification of SH-EP

SH-EP was purified from *V. mungo* cotyledons by an improved procedure based on the previous method [11]. Briefly, an extract of day-3 cotyledons (200 g) was fractionated by addition of ammonium sulfate (40% to 80% saturation). SH-EP was then separated from the precipitated protein by ion-exchange chromatography on a column (2 × 18 cm) of Whatman DE-52, which had been pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 10 mM ME. The loaded column was first washed with the buffer and subsequently eluted with a linear gradient (100 ml/100 ml) of 0 to 0.6 M KCl in the buffer. The active fractions were combined and concentrated by ammonium sulfate precipitation (80% saturation). SH-EP in the concentrated solution was further purified by gel filtration on a Sephacryl S-200 column followed by preparative electrophoresis in a polyacrylamide gel slab [11]. The enzyme protein recovered from the gel plate was dialyzed against water and lyophilized to a powder (0.5 mg).

### 2.3. Preparation of microsomes and protein bodies

The microsomal fraction was prepared essentially as described by Satoh and Fujii [15]. Day-3 cotyledons (25 g) were gently ground in a mortar and pestle with 62.5 ml of 0.2 M Tris-Cl buffer (pH 7.4) containing 0.44 M sucrose, 1 mM EDTA and 0.1 mM $MgCl_2$. 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 200,000 × *g* for 30 min, and the precipitate obtained was used as a microsomal fraction.

Protein bodies were prepared also from day-3 cotyledons by a modified method of Nishimura [12] and Nishimura and Beevers [13] and purified on a stepwise Percoll gradient. Sliced cotyledons (10 g) were incubated for 3 to 4 h at room temperature in a cellulase solution

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*Abbreviations:* CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; HPLC, high-pressure liquid chromatography; ME, 2-mercaptoethanol; MES, 2-morpholinoethanesulfonic acid; SDS, sodium dodecylsulfate.


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consisting of 20 mM MES-KOH (pH 5.5), 1% Cellulase Onozuka RS (Yakult), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical), 0.6 M mannitol, 1 mM $MgCl_2$ and 1% potassium dextran sulfate. The mixture was then filtered through nylon bolting cloth (224 mm) and centrifuged at 200 × *g* for 5 min. The precipitate was resuspended gently in the mannitol solution (20 mM MES-KOH, pH 5.5, containing 0.6 M mannitol and 1 mM $CaCl_2$) and allowed to stand for 2 h on ice. The supernatant solution containing 10% Percoll (Pharmacia) was layered on 25% Percoll in the mannitol solution and centrifuged at 7 × *g* for 20 min. The precipitate was resuspended gently in 0.5 to 1.0 ml of the mannitol solution, and the suspension was centrifuged again at 200 × *g* for 5 min. The precipitate obtained was used as a protein body fraction. Light-micrographic examination and assays of marker enzymes both indicated that the isolated protein bodies were intact and had negligible contamination of other organelles and cytoplasmic components [12,13].

*2.4. Immunological methods*

The preparation of antiserum to SH-EP and the protein immunoblotting were performed as described previously [11].

*2.5. CNBr cleavage of pyridylethylated SH-EP and isolation of the fragments*

An SH-EP preparation (0.3 mg protein) obtained as an active fraction from the Sephacryl S-200 column was pyridylethylated in 6 M guanidium hydrochloride [4]. The pyridylethylated SH-EP was then cleaved in 70% formic acid (0.20 ml) containing 2% (w/w) CNBr for 24 h at room temperature . After the reaction mixture was concentrated to about 50 μl under $N_2$ gas stream, it was applied to an octadecyl silica gel column (4.6 mm internal diameter × 25 cm; Shiseido), which was pre-equilibrated with 5% acetonitrile in 0.1% trifluoroacetic acid. After washing of the column with the same solvent, the resultant fragments were isolated by elution with a linear gradient (40 min) of 5 to 60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min.

*2.6. Analysis of amino acid composition and sequences*

The amino acid composition was determined with a Jasco A800 automated high performance amino acid analyzer equipped with a post-column derivatization system with *o*-phthalaldehyde. Peptides were hydrolyzed with 6 N HCl containing 5% (v/v) phenol at 110°C for 24 h in evacuated sealed tubes. The $NH_2$-terminal sequences of peptides produced by limited cleavage of pyridylethylated SH-EP were determined with an automated sequence analyzer (Model 477A, Applied Biosystems) with an on-line HPLC system for quantitative identification of phenylthiohydantoin derivatives of amino acids [9]. An active fraction of SH-EP obtained from the gel filtration column was electrophoresed in a 12.5% non-denaturing polyacrylamide gel, and the protein was electroblotted onto a PVDF membrane (Millipore) [5]. The region that included SH-EP was cut from the membrane, and the $NH_2$-terminal sequence was determined as described above.

*2.7. Mass spectroscopy*

Mass spectrum of an SH-EP preparation, which was obtained as an active fraction from the gel filtration column, was determined with a Finnigan Mat TSQ-700 mass spectrometer equipped with an electrospray ionization interface. The protein (1 μg) was introduced through a reversal phase $C_{18}$ capillary column (0.2 mm × 100 mm) connected in tandem to the electrospray ionization interface and the spectrometer. The resultant spectrum was analyzed by a computer-assisted software package provided by the supplier [9].

## 3. Results and discussion

*3.1. Intracellular localization of SH-EP and its precursor*

By SDS-polyacrylamide gel electrophoresis and protein immunoblotting with an antiserum made against SH-EP, the 43 kDa, 39 kDa and 36 kDa polypeptides, in addition to the 33 kDa polypeptide corresponding to mature SH-EP, were detected in an extract freshly prepared from cotyledons of day-3 *Vigna mungo* seedlings

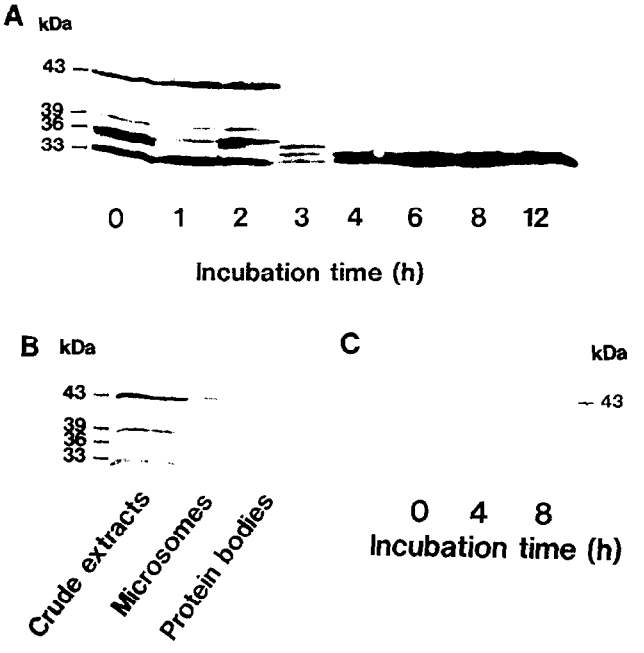


Fig. 1. Intracellular localization of 33 kDa mature SH-EP and its 43 kDa intermediate, and time course of processing of the intermediate. (A) Cotyledons (2 g) of day-3 *Vigna mungo* seedlings were homogenized with 6 ml of 50 mM Tris-Cl (pH 7.4) containing 10 mM ME. The homogenate was centrifuged at 25,000 × *g* for 20 min, and the supernatant solution was used as a crude extract. The extract was incubated for 0 to 12 h at 27°C, and at the time intervals indicated, the extract (0.2 mg each of protein) was analyzed by SDS-12.5% polyacrylamide gel electrophoresis and protein immunoblotting using an antiserum raised against SH-EP [11]. (B) Microsomal and protein body fractions were prepared from day-3 cotyledons as described in section 2. These fractions (0.2 mg each of protein) were analyzed as above. (C) The microsomal fracton (0.2 mg protein) was resuspended in 50 mM Tris-Cl buffer (pH 7.4) containing 10 mM ME, incubated for 0 to 8 h at 27°C and analyzed as above. The molecular masses indicated were estimated from the mobility on the SDS-gel electrophoresis.

(Fig. 1A). When the extract was allowed to stand at 27°C, the intensity of the 33 kDa polypeptide on the electrophoretic gel increased and other polypeptides became undetectable after 3 to 4 h of the incubation, and only the 33 kDa polypeptide was observed after 8 h (Fig. 1A). Only 43 kDa and 33 kDa polypetides were detected in microsomal and protein body fractions, respectively (Fig. 1B). When the microsomal preparation was incubated at 27°C for 8 h, no changes in the gel electrophoresis/immunoblot pattern were detected (Fig. 1C), suggesting the 43 kDa intermediate is processed further after being transported from the ER lumen. The results indicate that the 43 kDa intermediate of SH-EP formed in the ER lumen is transported to protein bodies and processed to the 33 kDa mature form during the transport to protein bodies or thereafter. These are coincident with our previous postulation that SH-EP together with a serine protease plays a major role in cotyledons of *V. mungo* seedlings in degrading storage globulin which has

been deposited in protein bodies during seed maturation [10].

### 3.2. COOH-terminal amino acid sequence of SH-EP

The amino acid sequence deduced from the nucleotide sequence of the SH-EP cDNA contains a KDEL sequence in its COOH-terminus [2]. The COOH-terminal tetrapeptide KDEL has been shown to be a signal for retention in the ER lumen of mammalian and plant cells [3,6,14,17], although immunological assays suggested that the COOH-terminal KDEL was retained on the auxin-binding protein found at the plasma membrane [7]. Recently Lee et al. [8] identified a cDNA clone from *Arabidopsis thaliana* as a homlogue to the ERD2 gene family, whose product is the receptor for the ER retention signal, and showed that this plant gene was able to complement a lethal phenotype of the yeast *erd2* deletion mutant. These facts are apparently inconsistent with the above postulation that SH-EP acts to degrade storage globulin which has been deposited in protein bodies. Our preliminary examinations with gel electrophoresis/immunoblotting have shown that a purified SH-EP preparation (10 μg) did not react with either of the mouse monoclonal antibodies 1D3 or 10C3 raised against the peptides KDDDKAVKDEL and KSEKDEL, respectively (generous gifts from Dr. S. Fuller, EMBL). We thus needed to determine whether or not the mature SH-EP has the COOH-terminal amino acid sequence containing a KDEL tail.

The direct $NH_2$-terminal sequence analysis (14 cycles of Edman degradation) of the mature SH-EP blotted to a PVDF membrane gave rise to a double sequence: $^{1}$Ser/Val-Val/Pro-Pro/Ala-Ala/Ser-$^{5}$Ser/Val-, etc., thereby suggesting that the mature SH-EP, as compared with the sequence of the precursor SH-EP deduced from its cDNA [2], contains two dominant species which are different by a single amino acid residue at the $NH_2$-terminus, one starting with Ser-127 and the other starting with Val-128.

The CNBr cleavage of pyridylethylated SH-EP resulted in four fragments (CB1 to 4), which were separated by reversed phase HPLC on a $C_{18}$ column. The amino acid composition and $NH_2$-terminal sequence analysis of these fragments indicate that each fragment covers residues 127/128–193 (CB1), 194–322 (CB2), 323–338 (CB3), and 339–352 (CB4). CB4 was a 14-residue fragment with the composition: $Asp_3$, Thr, $Ser_3$, $Pro_2$, Ala, Met, Ile, Tyr, Lys, and a sequence, Met-Asp-Ser-Tyr-Pro-Ile-Lys-Asn-Ser-Ser-Asp-Asn-Pro-Thr (Fig. 2), which corresponds to residues 339–352 in the precursor of SH-EP [2]. Because CB4 is only the fragment that contains neither homoserine nor its lactone at the COOH-terminus, we predicted that Thr-352 might be the COOH-terminus of the mature SH-EP.

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MAMKKLLWVVLSLSLVLGVANSFDFHEKDLESEESLWDLYERWRSH   46
HTVSRSLGEKHKRFNVFKANVMHVHNTNKMDKPYKLKLNKFADMTN   92
HEFRSTYAGSKVNHHKMFRGSQHGSGTFMYEKVGSVPASVDWRKKG  138
AVTDVKDQGQCGSCWAFSTIVAVEGINQIKTNKLVSLSEQELVDCD  184
KEENQGCNGGLMESAFEFIKQKGGITTESNYPYTAQEGTCDESKVN  230
DLAVSIDGHENVPVNDENALLKAVANQPVSVAIDAGGSDFQFYSEG  276
VFTGDCNTDLNHGVAIVGYGTTVDGTNYWIVRNSWGPEWGEQGYIR  322
MQRNISKKEGLCGIAMMASYPIKNSSDNPTGSLSSPKDEL        362
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Fig. 2. Amino acid sequence of SH-EP. The amino acid sequence of SH-EP deduced from the nucleotide sequence of the SH-EP cDNA is presented [2], and the amino acid sequence corresponding to peptides obtained by CNBr cleavage of the pyridylethylated SH-EP is underlined. Dots and asterisk indicate $NH_2$-terminal and COOH-terminal amino acids of 33 kDa mature SH-EP, respectively.

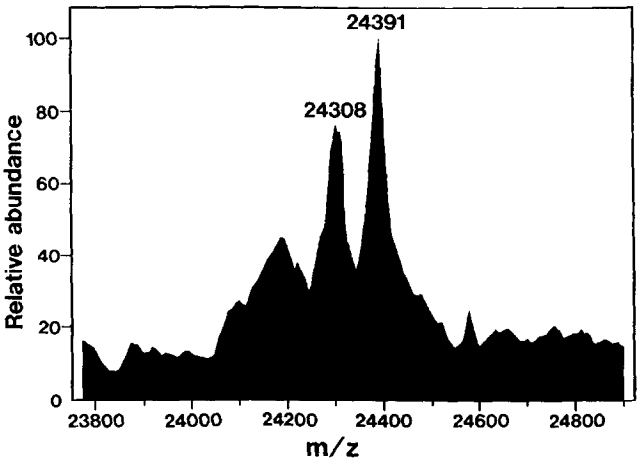


Fig. 3. Mass spectrum of purified SH-EP. The spectrum was reconstituted from the data recorded with electrospray mass spectrometry.

The mass spectrum of the purified SH-EP indicates the presence of two molecular species with masses, 24,308 and 24,391 (Fig. 3). These mass values are accounted for by the presence of two polypeptide chains, one extending from Val-128 to Thr-352 and the other extending from Ser-127 to Thr-352, in which the mass of each polypeptide is estimated from the sequence to be 24,310 and 24,397 (average mass value), respectively. We concluded, therefore, that the mature SH-EP has Thr-352 at the COOH-terminus and that the COOH-terminal decapeptide including a KDEL tail of the precursor protein has been removed by posttranslational processing during the maturation of SH-EP.

In the course of the sequence analyses we also noted that the mature SH-EP is a non-glycosylated protein, because three potential *N*-glycosylation sites, Asn-326, -346, and -350, were identified quantitatively by sequential Edman degradation and because the total mass of the mature SH-EP could be accounted for by the apopeptide sequence as described above.

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## References

[1] Akasofu, H., Yamauchi, D. and Minamikawa, T. (1990) Nucleic Acids Res. 18, 1892.
[2] Akasofu, H., Yamauchi, D., Mitsuhashi, W. and Minamikawa, T. (1989) Nucleic Acids Res. 17, 6733.
[3] Chrispeels, M.J. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 21–53.
[4] Friedman, M., Krull, L.H. and Cavins, J.F. (1970) J. Biol. Chem. 245, 3868–3871.
[5] Hirano, H., Komatsu, S., Takakura, H., Sakiyama, F. and Tsunasawa, S. (1992) J. Biochem. 111, 754–757.
[6] Inohara, N., Shimomura, S., Fukui, T. and Futai, M. (1989) Proc. Natl. Acad. Sci. USA 86, 3564–3568.
[7] Jones, A.M. and Herman, E.M. (1993) Plant Physiol. 101, 595–606.
[8] Lee, H.-I., Gal, S., Newman, T.C. and Raikhel, N.V. (1993) Proc. Natl. Acad. Sci. USA 90, 11433–11437.
[9] Matsuoka, K., Seta, K., Yamakawa, Y, Okuyama, T., Shinoda, T. and Isobe, T. (1994) Biochem. J. 298, 435–442.
[10] Mitsuhashi, W., Koshiba, T. and Minamikawa, T. (1986) Plant Physiol. 80, 628–634.
[11] Mitsuhashi, W. and Minamikawa, T. (1989) Plant Physiol. 89, 274–279.
[12] Nishimura, M. (1982) Plant Physiol. 70, 742–744.
[13] Nishimura, M. and Beevers, H. (1978) Plant Physiol. 62, 44–48.
[14] Pelham, H. (1988) EMBO J. 7, 913–918.
[15] Satoh, S. and Fujii, T. (1985) Plant Cell Physiol. 26, 1037–1044.
[16] Yamauchi, D., Akasofu, H. and Minamikawa, T. (1992) Plant Cell Physiol. 33, 789–797.
[17] Warren, G. (1987) Nature 327, 17–18.