Activation of Arabidopsis Vacuolar Processing Enzyme by Self-Catalytic Removal of an Auto-Inhibitory Domain of the C-Terminal Propeptide

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Vacuolar processing enzyme (VPE) is a cysteine proteinase responsible for the maturation of various vacuolar proteins in higher plants. To clarify the mechanism of maturation and activation of VPE, we expressed the precursors of Arabidopsis γVPE in insect cells. The cells accumulated a glycosylated proprotein precursor (pVPE) and an unglycosylated preproprotein precursor (ppVPE) which might be unfolded. The N-terminal sequence of pVPE revealed that ppVPE had a 22-amino-acid signal peptide to be removed co-translationally. Under acidic conditions, the 56-kDa pVPE was self-catalytically converted to a 43-kDa intermediate form (iVPE) and then to the 40-kDa mature form (mVPE). N-terminal sequencing of iVPE and mVPE showed that sequential removal of the C-terminal propeptide and N-terminal propeptide produced mVPE. Both iVPE and mVPE exhibited the activity, while pVPE exhibited no activity. These results imply that the removal of the C-terminal propeptide is essential for activating the enzyme. Further removal of the N-terminal propeptide from iVPE is not required to activate the enzyme. To demonstrate that the C-terminal propeptide functions as an inhibitor of VPE, we expressed the C-terminal propeptide and produced specific antibodies against it. We found that the C-terminal propeptide reduced the activity of VPE and that this inhibitory activity was suppressed by specific antibodies against it. Our findings suggest that the C-terminal propeptide functions as an auto-inhibitory domain that masks the catalytic site. Thus, the removal of the C-terminal propeptide of pVPE might expose the catalytic site of the enzyme.

Key words: Auto-inhibitory domain — Cysteine proteinase — Propeptide — Protein maturation — Vacuolar processing enzyme — Vacuole.

Abbreviations: VPE, vacuolar processing enzyme; ppVPE, preproprotein precursor; pVPE, proprotein precursor; iVPE, intermediate form of VPE; mVPE, mature form of VPE; gV-C, C-terminal propeptide of γVPE.

Introduction

Vacuolar processing enzyme (VPE) was originally found as a novel cysteine proteinase responsible for maturation of seed storage proteins (Hara-Nishimura et al. 1991a). In vitro processing assays with the purified VPE have demonstrated that VPE is the essential processing enzyme mediating maturation of several seed proteins of pumpkin and castor bean, including 2S albumins (Hara-Nishimura et al. 1991a, Hara-Nishimura et al. 1993a), 11S globulins (Hara-Nishimura 1987, Hara-Nishimura and Nishimura 1987, Fukasawa et al. 1988), 7S globulin (Yamada et al. 1999), ricin and Ricinus communis agglutinin (Hiraiwa et al. 1997a). VPE recognizes exposed asparagine residues on the molecular surface of precursor proteins and then cleaves the peptide bonds at the carbonyl sides of the asparagine residues (Hara-Nishimura et al. 1993a, Hara-Nishimura 1998). Recently, we found that VPE cleaves Asn-Gln bonds of a single precursor to produce multiple functional proteins including a trypsin inhibitor, cytotoxic peptides and a vicilin-like storage protein (Yamada et al. 1999). We also found that VPE might be responsible for maturation of a novel membrane protein MP 32 (Inoue et al. 1995). Most of the known seed proteins of different plants are produced by proteolytic cleavages at the asparagine, as reviewed by Hara-Nishimura et al. (1995). Not only seed proteins but also some vacuolar proteins of leaves have a possible processing site to be cleaved by VPE (Hara-Nishimura et al. 1998a, Kinoshita et al. 1999). Therefore, VPE homologs could be distributed in a wide range of plant species to mediate the maturation of vacuolar proteins in seeds and vegetative organs.

Various cDNAs of VPE homologs have been isolated from both maturing seeds (Hara-Nishimura et al. 1993b, Shimada et al. 1994, Takeda et al. 1994) and vegetative organs (Becker et al. 1995, Becker et al. 1997, Alonso and Granell 1995). We have isolated three genomic clones for Arabidopsis VPE, αVPE, βVPE and γVPE (Kinoshita et al. 1995a, Kinoshita et al. 1995b). These homologs can be separated into two subfamilies: one specific to seeds and the other specific to vegetative organs. This is consistent with the fact that the plant vacuoles are classified into two types, protein-storage vacuoles and lytic vacuoles. The vegetative VPE is localized in the lytic
vacuoles (Kinoshita et al. 1999), while the seed VPE is localized in the protein storage vacuoles (Hiraiwa et al. 1993). These findings suggest that αVPE and γVPE play a role in the maturation of some proteins in the lytic vacuoles of the vegetative organs of Arabidopsis, while βVPE is involved in the maturation of the seed proteins in the protein-storage vacuoles.

The vegetative VPE is up-regulated in the lytic vacuoles during senescence and under various stress conditions (Kinoshita et al. 1999). Recently we found that, in young seedlings, the vegetative VPE is specifically accumulated in a novel compartment, the ER bodies of the epidermal cells, which are easily wounded and stressed by the external environment (Hayashi et al. 2001). When the seedlings are exposed to stress leading to death of the epidermal cells, the ER bodies start to fuse with the vacuoles. Thus, VPE might assist in cell death when the plant is exposed to stress. A barley homolog of VPE, nucellain, is reported to be localized in nucellar cell walls which are known to be degenerated in developing cereal grains (Linnestad et al. 1998, Dominguez et al. 2001). They suggested that nucellain plays a role in processing and/or turnover of cell wall proteins. These results suggest that the vegetative VPEs are responsible for maturation of lytic enzymes that function in programmed cell death of higher plants.

Arabidopsis γVPE could be synthesized as a larger precursor (Kinoshita et al. 1995b). This raises the question of whether the γVPE precursor is inactive and, if so, how the precursor is converted into the active form. Previously we reported that the seed VPE of castor bean is self-catalytically converted into the mature form (Hiraiwa et al. 1997b, Hiraiwa et al. 1999). However, the detailed mechanism for the conversion of the vegetative VPE remains obscure. In mammals, the mechanism of maturation of proteinases has been well characterized with cathepsins and caspases. Cathepsin B, K, L and S are synthesized as latent precursors that are subsequently converted into the active enzymes by limited proteolysis of the N-terminal propeptide (Mach et al. 1993, McQueney et al. 1997, Menard et al. 1998, Maubach et al. 1997). The N-terminal propeptide of cathepsins serves as a regulator of catalytic activity. Procaspsases are known to be converted into the active form by a self-catalytic removal of a linker peptide (Cohen 1997, Stennicke and Salvesen 1998).

To clarify the VPE-mediated processing system in the lytic vacuoles, it is necessary to explore the detailed mechanism of the maturation and activation of vegetative VPE. Here, we demonstrate that the C-terminal propeptide of γVPE functions as an auto-inhibitory domain of the enzyme, and show that the self-catalytic removal of the C-terminal propeptide is required to generate an active enzyme.

Results

Expression of precursors of Arabidopsis VPE in insect cells

We expressed the precursors of Arabidopsis γVPE in insect cells (SF-21) with a baculovirus system (Fig. 1A). Two major proteins of 56 kDa and 55 kDa were found in the extract from the transformant cells. An N-terminal sequence analysis showed that the 56-kDa protein corresponded to proprotein precursor of VPE (pVPE) (Fig. 1B). The result revealed that the N-terminal 22-amino-acid sequence corresponded to a sig-

![Fig. 1](image-url)
nal peptide to be removed co-translationally. The cleavage site was consistent with the predicted site by application of the rule of von Heijne (von Heijne 1986). The N-terminal sequence of the 55-kDa protein was consistent with the sequence starting from the second amino acid (Thr-2) of the precursor. The 55-kDa protein might be the preproprotein precursor of VPE (ppVPE) that contained no first Met.

The VPE precursor has a possible glycosylation site on the polypeptide (Fig. 7B). When both ppVPE and pVPE were subjected to Con A-column chromatography, pVPE was bound to the column, but ppVPE was not (Fig. 1C, upper). This indicated that pVPE had an N-linked oligosaccharide, but ppVPE did not. The glycosylation of pVPE was also supported by the evidence that the molecular mass (56 kDa) of pVPE was reduced to be 53 kDa by the treatment with N-glycosidase F (Fig. 1C, lower). These findings indicated that pVPE was produced by co-translational cleavage of the signal peptide and glycosylation on rough endoplasmic reticulum (rER). By contrast, ppVPE might be abnormally accumulated in the cytosol. To compare the stability of ppVPE with pVPE, both proteins were incubated for 30 min at pH 7.5 and 37°C. Fig. 2 shows that ppVPE was rapidly degraded, but pVPE was not. This suggested that ppVPE was unfolded and pVPE was folded correctly in the insect cells.

Fig. 2  No conversion of pVPE into the mature form (mVPE) under the neutral condition. Both ppVPE and pVPE that were extracted from the transformant cells were incubated in the neutral buffer (pH 7.5) for 0 (lane 1) and 30 (lane 2) min at 37°C. The VPEs were subjected to SDS-PAGE followed by immunoblotting with anti-γVPE antibodies.

Self-catalytic conversion of pVPE into an intermediate (iVPE) is accompanied by activation of the enzyme

To explore the mechanism of maturation of VPE, the recombinant pVPE was incubated in an acidic buffer (pH 5.5). During the incubation of 30 min, the 56-kDa pVPE was self-catalytically converted to a 43-kDa intermediate form (iVPE) and then to the 40-kDa mature form (mVPE), as shown in Fig. 3A. The conversion of pVPE at pH 5.5 did not occur at pH 7.5 (Fig. 2). The pH-dependent conversion of pVPE into iVPE occurred in a self-catalytic manner. This is consistent with the evidence that γVPE (data not shown) and seed VPE (Hayashi et al. 1988) exhibited maximum activity at pH 5.5 and no activity at pH 7.5. iVPE was also self-catalytically converted into mVPE by incubation for 30 min at pH 5.5 (Fig. 3B).

To answer the question of whether the conversion is accompanied by activation of the enzyme, we prepared pVPE, iVPE and mVPE from the transformant cells to measure each specific activity. The extract from insect cells infected with wild-type baculovirus had no activity (data not shown). Fig. 4A shows that pVPE had no activity at all, but both iVPE and mVPE did. It is noted that iVPE (1.8 U (mg protein)−1) had specific activity at a level similar to that of mVPE (2.1 U (mg protein)−1). The results indicate that the conversion of pVPE into iVPE is in association with the activation of the enzyme.

Previously we reported that seed VPE purified from castor bean is not a glycoprotein and has specific activity of 1.9 U (mg protein)−1 (Fig. 4A) (Hara-Nishimura et al. 1991a). This raised another question of whether the deglycosylation of pVPE is responsible for the activation of the enzyme. To answer this, both iVPE and mVPE were subjected to Con A-column chromatography. Fig. 4B showed that both iVPE and mVPE were specifically bound to the column and that both have an N-linked oligosaccharide. The result indicated that deglycosylation did not occur during the conversion and that the glycosylation did not affect the enzyme activity.

Fig. 3  Two-step-conversion of pVPE into mVPE via an intermediate (iVPE) under an acidic condition. (A) pVPE that was extracted from the transformant cells with a neutral buffer (pH 7.5) was incubated in an acidic buffer (pH 5.5) for 0 to 30 min (lanes 1–6) at 37°C. Each sample was subjected to SDS-PAGE followed by an immunoblot with γVPE-antibodies. pVPE was converted into the intermediate form (iVPE) and then the mature form (mVPE) under an acidic condition. Asterisk indicates a degradation product. (B) The transformant cells were homogenized in an acidic buffer (pH 5.5) to obtain both iVPE and mVPE. The VPEs were incubated in an acidic buffer (pH 5.5) for 0 to 30 min (lanes 1–6) at 37°C. The following procedures were the same as described in (A).
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Fig. 4  pVPE has no VPE activity, while both iVPE and mVPE have activity. (A) Specific activity of pVPE, iVPE and mVPE was measured. The specific activity of seed VPE of castor bean (*) was obtained referring to (1). (B) Both iVPE and mVPE that were extracted as described in Fig. 3B were subjected to Con A-column chromatography as described in Fig. 1C. Each fraction was subjected to SDS-PAGE followed by immunoblotting with anti-iVPE antibodies.

**Fig. 5**  iVPE has a 24-amino-acid N-terminal propeptide, but no C-terminal propeptide. The molecular structure of iVPE and mVPE. (A) The determined N-terminal sequences of iVPE and mVPE are underlined. The cleavage site of the N-terminal propeptide is at the C-terminal side of Asn-46 (N). (B) Either the extract containing ppVPE and pVPE (lanes 1 and 2) or the extract containing iVPE and mVPE (lanes 3 and 4) was subjected to SDS-PAGE followed by immunoblotting with either anti-iVPE antibodies (lanes 1 and 3) or anti-gV-C antibodies (lanes 2 and 4).

**Activation of VPE is caused by the cleavage of the C-terminal propeptide**

To clarify the molecular structures of iVPE and mVPE, we determined the N-terminal sequences. Fig. 5A shows that the sequence of the 43-kDa iVPE was the same as that of the 56-kDa pVPE. The result implied that the 13-kDa C-terminal propeptide should be removed from inactive pVPE to produce active iVPE. This self-catalytic removal of the C-terminal propeptide may be required for activating the enzyme. On the other hand, the N-terminal sequence of mVPE showed that the...
N-terminal 24-amino-acid propeptide was cleaved off at the C-terminal side of Asn-46 (Fig. 5A). The cleavage site is consistent with the substrate specificity of γVPE (Kinoshita et al. 1999). Self-catalytic removal of the N-terminal propeptide to produce mVPE was also supported by the result shown in Fig. 3. These results indicate that sequential removal of the C-terminal propeptide and that the N-terminal propeptide is necessary to make the mature mVPE.

To explore the function of the C-terminal propeptide in detail, we produced specific antibodies against a C-terminal propeptide from Lys-412 to Ala-490 that was designated gV-C (discussed below). Both ppVPE and pVPE were prepared from the transformant cells at pH 7.5, while both iVPE and mVPE were prepared from the cells at pH 5.5 (Fig. 3). These VPEs were immunoblotted with anti-gV-C antibodies. The inactive forms of ppVPE and pVPE had the C-terminal propeptide, but the active forms of iVPE and mVPE did not (Fig. 5). This was in agreement with the above results. These findings indicate that the activation of VPE is caused by cleavage of the C-terminal propeptide, but not that of the N-terminal propeptide.

The C-terminal propeptide functions as an auto-inhibitory domain that masks the catalytic site

The next issue to be resolved was whether the C-terminal propeptide functions as an inhibitory domain. The processing site for the 13-kDa C-terminal propeptide was deduced to be at the C-terminal side of Asn-411, which was consistent with the substrate specificity of γVPE (Kinoshita et al. 1999). Therefore, the C-terminal propeptide was predicted to be the region from Lys-412 to Ala-490 and was designated gV-C. To clarify the effect of gV-C on the VPE activity, we measured the inhibitory activity of the expressed gV-C with the fluorescent substrate. The fluorescent intensity showing the VPE activity was reduced along with the increase of the amount of the gV-C protein in the reactions. The extract from non-transformed E. coli had no effect on the VPE activity (Fig. 6A, upper). Fig. 6A (lower) represents the results quantitatively.

To confirm that the reduction of the VPE activity was caused by gV-C, we examined the effect of addition of anti-gV-C antibodies on the inhibitory activity of gV-C. Fig. 6B shows that the inhibitory activity was suppressed by the addition of specific antibodies against gV-C. We calculated that one molecule of mVPE stoichiometrically interacted with one molecule of gV-C. These results indicate that the C-terminal propeptide of VPE functions as an auto-inhibitory domain to mask the VPE active center and inhibit the activity.

Discussion

Fig. 7A shows the hypothetical process of maturation and activation of VPE in the lytic vacuoles of higher plants. pVPE synthesized on rER is transported to acidic compartments, the lytic vacuoles, where the C-terminal propeptide that masks the active site of the enzyme is self-catalytically removed from pVPE to generate the active iVPE. Subsequent removal of the N-terminal propeptide at the C-terminal side of Asn-46 produces mVPE. The processing at asparagine is mediated by VPE itself, which has a substrate specificity towards asparagine residues. In contrast to the processing site at an asparagine residue,
the site for seed VPEs has been reported to be at an aspartic acid (Becker et al. 1995, Hiraiwa et al. 1999).

We demonstrated that the C-terminal propeptide of VPE functions as an auto-inhibitory domain. The removal of the C-terminal propeptide was coupled with activation of VPE. Chen et al. (2000) reported that a synthetic partial peptide (Ac-KDLEESRQLTEEIQRHD) from the C-terminal propeptide of a human homolog of VPE, legumain, slightly inhibited the pig legumain activity ($K_i = 630 \mu M$). The present study showed that the 13-kDa gV-C domain bound stoichiometrically to the mVPE protein and inhibited the enzyme activity (Fig. 6). However, the gV-C domain does not have a sequence similar to the above peptide (Fig. 7B). Such activation mechanism by removal of a C-terminal propeptide is not known for other proteinases. Recently, we found that Arabidopsis RD21, a cysteine proteinase of the papain family, was accumulated in the vacuoles as an aggregate and then slowly matured to make a soluble proteinase by removing the C-terminal extension sequence homologous to animal epithelin/granulin (Yamada et al. 2001).

In mammals, lysosomal cathepsins were reported to have an N-terminal propeptide that acts as an auto-inhibitory domain (Maubach et al. 1997, Cysler and Mort 1997, Quraishi et al. 1999, Carmona et al. 1996, Coulombe et al. 1996, Kessler and Safrin 1994, Serkina et al. 1999). The inactive precursor of cathepsin L is converted into the fully active mature enzyme by removing the N-terminal propeptide of 96 amino acids (Mason et al. 1989). On the other hand, iVPE that had the N-terminal propeptide of 24 amino acids exhibited full activity (Fig. 4). Thus, the N-terminal propeptide of VPE does not affect the activity. Then, what is its physiological function? The N-terminal propeptide of cathepsin L has been shown to be required for proper folding of the protein (Tao et al. 1994) and for targeting to the lysosomes (McIntyre et al. 1994). It is possible that the N-terminal propeptide of VPE has a function similar to that of cathepsin L.

VPE might be transported to the vacuoles as an inactive pVPE, which is converted into an active enzyme, iVPE, and then into mVPE under an acidic condition. It has been shown that an acidification causes self-catalytic activation of various proteinases (McQueney et al. 1997, Menard et al. 1998, Mach et al. 1994, Bromme et al. 1993, Kageyama et al. 1992, Van Den Hazel et al. 1997). Acidic-pH-dependent maturation might be a common feature for activation of both lysosomal proteinases in animals and vacuolar proteinases in plants. The proteinases must be converted into mature forms only when they reach the compartments where they are to act. Both pig legumain and mouse legumain that are active at an acidic pH are irreversibly inactivated at pH 7.0 (Chen et al. 1997, Chen et al. 1998). By contrast, pVPE is stable at pH 7.5 (Fig. 2). Procathepsin L is also stable at the alkaline pH (Nomura and Fujisawa 1997). The N-terminal propeptide prevents the protein from denaturing at alkaline pH (Nomura and Fujisawa 1997).

Leakage of lysosomal proteinases, cathepsins, into the cytosol has been suggested to be involved in the activation of caspases (Ishisaka et al. 1998). Both cathepsin B and cathepsin D leak out from the lysosomes to be one of the executors of apoptotic pathways during the cell death, like caspases (Li et al. 1998, Roberg and Ollinger 1998). These proteinases could be regulated by their own auto-activation preventing inappropriate activation that causes a fatal event to the cell. Self-catalytic activation may be a common feature of the proteinases related to cell death. We found that pVPE is self-catalytically converted into the mature form (Fig. 3), as seed VPE is (Hiraiwa et al. 1997b). Such self-catalytic conversion has also been reported with caspase-2, 8 and 9 (Ahmad et al. 1997, Boldin et al. 1996, Muzio et al. 1996, Srinivasula et al. 1998).

Recently, we found that stress-inducible proteinases, VPE and RD21, are localized in the novel compartment, designated ER bodies, which is derived from endoplasmic reticulum in the epidermal cells of Arabidopsis seedlings. The ER bodies might store inactive precursors of these enzymes that are ready to be transported to act in the vacuoles and finally within the dead cells. In the vacuoles, the components are thought to be converted to the active molecules.

The mechanism of cell death in animals and plants has been thought to share common components. However, the way in which dying cells are degraded in plants is different from that in animals. Because plants do not have macrophages, plant cells must degrade their materials by themselves during senescence and under various stresses. However, wounded and/or stressed cells would not be able to synthesize hydrolytic enzymes to degrade cellular materials. In most cases of plant programmed cell death, vacuolization of the cytoplasm and disruption of the tonoplast are common events (Fukuda 1996, Groover and Jones 1999, Lam et al. 1999). Therefore, VPE plays a crucial role in programmed cell death of plants.

**Materials and Methods**

**Expression of Arabidopsis VPE in insect cells**

Arabidopsis γVPE was produced employing the baculovirus expression system (Invitrogen, San Diego, CA, U.S.A.). The entire reading frame of the γVPE cDNA (1473 bp) was inserted into a transfer vector pBlueBac4.5 and cotransfected together with linearized Bac-N-Blue DNA (baculoviral Autographa californica multiple polyhedrosis viruses) in the cells of Spodoptera frugiperda (SF-21). The following procedures were the same as described before (Hayashi et al. 1999). The log-phase growing SF-21 cells were infected with the recombinant virus to obtain a large-scale expression of the protein.

**Extraction of recombinant proteins and determination of N-terminal sequences**

Two days after infection, the cells were collected by centrifugation at 750×g, 4°C for 5 min and were washed twice with phosphate-buffered saline (PBS). The cells were gently suspended in either neutral buffer A (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride) or acidic buffer B (50 mM sodium acetate pH 5.5, 50 mM NaCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 1 h, the cell suspensions were lysed by three bursts of sonication. After centrifugation
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at 15,000×g for 30 min, the supernatant was used for the experiments.

The supernatant was subjected to SDS-PAGE and then blotted onto a polyvinylidene difluoride membrane (0.22 μm; Nihon Millipore, Tokyo Japan). The bands corresponding to γVPE were cut out from the blot and subjected to automatic Edman degradation on a peptide sequencer (model 494, PerkinElmer/Applied Biosystems, Foster City, CA, U.S.A.).

Concanavalin A (Con A)-column chromatography

Both ppVPE and pVPE were extracted with buffer A, and both iVPE and mVPE were extracted with buffer B as described above. CaCl\(_2\) (final concentration of 10 mM) was added to the extracts. Each extract (0.2 mg VPEs) was applied to a Con A–agarose column (10 ml of a bed volume; Honen Corp., Tokyo, Japan), followed by washing with 50 ml of the respective buffer, with 30 ml of the buffer containing 500 mM NaCl, and then with 20 ml of the buffer. Finally, bound proteins were eluted by the addition of 1 ml of each buffer containing 200 mM α-methyl-mannoside. Running buffers used contained no EDTA.

Expression of C-terminal propeptide of gVPE and assay for inhibitory activity

The putative C-terminal propeptide (gV-C) of Arabidopsis γVPE was predicted to be amino acids 412–491. A cDNA encoding the propeptide was inserted into pQE 30 (Qiagen, Tokyo, Japan) and E. coli was transformed with the construct. The His-tagged gV-C was purified with a HiTrap™ chelating column. The purified protein was used for production of specific antibodies (described below) and for assay of inhibitory activity.

For inhibitory assay of gV-C, we synthesized a fluorescent VPE-specific substrate, Ac-ESN-(4-Metyl-Coumaryl-7-Amide); Peptide Institute, Inc., Osaka, Japan. The recombinant VPE was incubated in an acidic buffer (100 mM sodium acetate pH 5.5, and 100 mM dithiothreitol) for at least 8 min at 37°C to obtain an active enzyme. After addition of gV-C, the enzyme solution was further incubated for 8 min. The substrate (100 μM) was added to the reaction solution. The reaction mixture (100 μl) was incubated at 37°C. The fluorescent image was analyzed with Image Master VDS-CL (Amersham Pharmacia Biotech) and the fluorescence intensity was measured using GENios (TECAN, Männedorf, Switzerland) for kinetics analysis. The fluorescence was monitored under an excitation wavelength, 390 nm and an emission wavelength, 460 nm. The fluorescence intensity represents the VPE activity.

To examine the ability of gV-C as the inhibitory domain, gV-C was treated with anti-gV-C antibodies at each concentration. The mixture was pre-incubated for 8 min with mVPE. After centrifugation, the supernatant was collected. The reaction started by addition of Ac-ESEN-MCA and the fluorescence intensity was measured using GENios (TECAN, Männedorf, Switzerland) for kinetics analysis. The fluorescence was monitored under an excitation wavelength, 390 nm and an emission wavelength, 460 nm. The fluorescence intensity represents the VPE activity.

Production of specific antibodies

A cDNA encoding the region of γVPE (amino acids 76–213) was inserted into pQE 30 vector (Qiagen) and E. coli was transformed with the plasmid. The His-tagged protein was purified with a HiTrap™ chelating column. The purified protein was used for production of specific antibodies against γVPE.

Each of the purified protein (1 mg) and the purified gV-C (2 mg) in 1 ml of PBS was emulsified with an equal volume of Freund’s complete adjuvant. Each emulsion was injected subcutaneously into a rabbit. After 3 weeks, two booster injections with incomplete adjuvant were given at 7-d intervals. One week after the booster injection, blood was drawn and the antiserum was prepared. The antibodies prepared against γVPE were purified using a Mab TrapTM G II column (Amerham Pharmacia Biotech).

Immunoblot analysis

The recombinant VPEs were subjected to SDS-PAGE and were transferred electrophoretically to a PVDF. The following procedures were essentially as described previously (Mitsuhashi et al. 2000). The blotted membrane was incubated with either antibodies against the region of γVPE (anti-γVPE antibodies, diluted 2,500-fold) or antibodies against the putative C-terminal propeptide of γVPE (anti-gV-C antibodies, diluted 5,000-fold). Horseradish peroxidase-conjugated donkey antibodies against rabbit IgG (diluted 5,000-fold; Amersham Pharmacia Biotech) were used as second antibodies. VPEs were visualized with an enhanced chemiluminescence kit (an ECL system; Amerham Pharmacia Biotech).

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