Mutational Analysis of the Amino Acid Residues Essential for the *cis* and *trans* Cleavage Activity of the Potato Virus Y 50-kDa Protease

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To examine the proteolytic activities of various truncated derivatives of the potato virus Y (PVY) 50-kDa protease, the derivatives were expressed in *Escherichia coli* in polyprotein forms fused with coat protein (CP). For the intermolecular cleavage reaction, the truncated proteases were expressed together with the substrate protein containing the polymerase–CP junction. The activity was evaluated by the amount of the mature CP released from the precursor by the intra- and intermolecular cleavage occurring in *E. coli*. By this experiment, we identified the moiety responsible for the proteolytic activity of the 50-kDa protease to be a 26-kDa polypeptide mapped to the C-terminal half of the protease. Introduction of His234→Tyr, Asp269→Asn, or Cys339→Gly substitution in the putative catalytic triad of the protease abolished its activity. However, the mutated protease with Cys339→Ser replacement retained a reduced proteolytic activity.

Potato virus Y (PVY) is the type member of the potyvirus group, the largest plant virus group, arranged in the picorna-like supergroup. The potyviruses have positive-sense single-stranded RNA genomes containing a single open reading frame that encodes a large polyprotein. This polyprotein precursor is then processed to a coat protein and at least eight viral non-structural proteins by its own protease activity (reviewed by Riechmann et al.2).

In our previous paper,3) we showed that a 50-kDa protein within the PVY polyprotein is a protease responsible for the excision of coat protein from the polyprotein. The N-terminal 62-amino acid truncation of the PVY protease did not affect its proteolytic activity. In the case of tobacco etch virus (TEV), a potyvirus, the proteolytic activity is associated with the 49-kDa small nuclear inclusion (Nia) protein.4) This Nia protein itself is a multifunctional polyprotein and the proteolytic activity has been mapped to the C-terminal half of Nia protein.5,6) The N-terminal half serves as the genome-linked protein (VPg). The amino acid sequence of the PVY protease shows approximately 50% homology with that of the TEV Nia protein.3) From these aspects, it is predicted that the N-terminal half of the PVY protease should be dispensable for the proteolytic activity.

Bazan and Fletterick7) and Gorbalenya et al.8) independently proposed that the proteases encoded by the viruses belonging to the picorna-like supergroup are related to trypsin-like serine proteases. The major difference between the trypsin-like protease and viral proteases is that the nucleophilic serine residue constituting the catalytic triad of the former is replaced by cysteine in the latter. In the case of the TEV Nia protease, it has been proved that the catalytic triad is composed of histidine, aspartic acid, and cysteine residues at amino acid positions 234, 269, and 339, respectively.9) As the candidates constituting the catalytic triad of the PVY protease, Bazan and Fletterick proposed His234, Asp269, and Cys339 according to the alignment of the amino acid sequences of both proteases.10) However, the presence of a histidine residue at the 233rd position of the PVY protease raises a question as to which histidine residue (His233 or His234) is responsible for the proteolytic activity. To analyze the catalytic triad residues experimentally, we prepared some mutated PVY proteases with amino acid substitutions at the predicted three residues and some other histidine, aspartic acid, and cysteine residues and tested their proteolytic activities.

In this paper, we examined not only the intramolecular (*cis*) but also the intermolecular (*trans*) cleavage activity of the PVY protease by expressing the protease and its substrate protein in the same *Escherichia coli* cells. Our results of the truncation and site-directed mutagenesis analyses indicated that the proteolytic activity was mapped to the C-terminal half of the PVY protease and that the histidine residue constituting the catalytic triad was His234, and not His233. The other components were Asp269 and Cys339, confirming the prediction of Bazan and Fletterick.10) The substitution of Cys339 by a serine residue did not abolish the proteolytic activity of the PVY protease. The mutated protease possessed reduced activity.

Materials and Methods

Construction of plasmids. The construction of pPVY2 and pPVY4 is described in our previous paper.11) Both plasmids code for the PVY polyprotein consisting of the PVY protease and coat protein (CP), but the polyprotein encoded by pPVY4 has a 14-amino acid deletion overlapping the putative active site residue of the protease. A series of plasmids coding for the N-terminally truncated PVY proteases was constructed as follows. The position of each restriction site is indicated as the nucleotide position (nt) of the PVY cDNA that we cloned previously.11) The PvuII (1527 nt) to PstI (downstream of the 3' end of PVY cDNA fragment) portion of pPVY2 was prepared. The insertion of this fragment between the XhoI (filled-in) and PstI sites of pUC18 made pPVY2-171. By the subcloning of the fragment after chopping its PvuII end region with exonuclease III, plasmids pPVY2-193, -198, -201, and -231 were constructed. The 5' end regions of the chewed fragments were identified by sequencing. The gene products expressed from these plasmids start from the 171st, 193rd, 198th, 203rd, and 231st amino acid of the PVY protease, respectively, with several preceding amino acids derived from the N-terminal region of β-galactosidase. A plasmid pPVY3 was made by deleting the AarI–SspI (2466–4767 nt) portion of pPVY1.13) By this construction, the open reading frame encoding the PVY protein ends 168 nucleotides (coding for 56 amino acids) downstream of the C-terminal position of the PVY protease. Plasmids pPVY3-C10 and pPVY3-C33 were constructed by inserting a stop codon (Ochre)/HpaI linker (Pharmacia) into the BglII (2280 nt,
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Fig. 1. Schematic Representation of Plasmids Containing PVY cDNA Inserts.

The designed structure of polyprotein encoded by the PVY cDNA insert contained in pPVY2 is illustrated at the top. The large box indicates the open reading frame (ORF), while vertical lines in the box represent the internal cleavage sites of the PVY polyprotein. A polypeptide between protease (Pro) and coat protein (CP) represents polymerase (Pol) lacking a large portion of its middle region. A shadowed box in each plasmid represents the region actually translated in E. coli.

Analysis of PVY protein expressed in E. coli. E. coli GC4670 (lon::Tn 10 thr leu lacY) cells harboring the PVY protease-expressing plasmids of pUC18 derivatives were grown in L broth containing 50 μg/ml ampicillin. For the cultivation of E. coli cells harboring the pACYC derivatives, 30 μg/ml chloramphenicol was added to the media. At the mid-log phase of the cultivation (OD 650 nm is 0.5), isopropyl-β-D-thiogalactoside (IPTG) was added to the culture at the concentration of 2 mM and the culture was incubated for 6 h more. The cells were harvested, disrupted by sonication, and centrifuged at 8000 x g for 10 min. The precipitate was recovered and analyzed by Western blotting using anti-PVY CP antibody and anti-PVY protease antibody as described in our previous paper.3)

Site-directed mutagenesis. The site-directed mutagenesis of pPVY2 were designed to exchange the histidine, aspartic acid, and cysteine residues with tyrosine, asparagine, and glycine, respectively. In the case of the mutagenesis of Cys339, the replacement of the cysteine residue with serine was also designed. The mutagenesis were done by the method of Kunkel et al.11 using 5'-TAGGGAACACATTTGT-3', 5'-CGAACACTATTGGTCT-3', 5'-GATCCATGTACCTCA-3', 5'-AGAATCTATACGTGTG-3', 5'-AAGGTAAGCAGCATAC-3', 5'-TGCCAAAAATACTCCT-3', 5'-AAAGAATTTGTTATGTTT-3', 5'-ATGGACACAGTGGACAT-3', 5'-ATGGACACGCTGGACTG-3', and 5'-CCGATGGACGCTGTGCT-3' as the synthesized mutagenic oligonucleotides. The constructed plasmids were designated pPVY2-H233Y, pPVY2-H234Y, pPVY2-H249Y, pPVY2-H258Y; pPVY2-D269N, pPVY2-D278N, pPVY2-C298G, pPVY2-C339S, pPVY2-C339G, and pPVY2-C350G, respectively. The notations after pPVY2 indicate the position of the replaced amino acid residues (e.g., H233Y indicates that His233 is replaced by tyrosine).

Results and Discussion

The N-terminal half of the PVY protease is not needed for its proteolytic activity

We have reported20 that the PVY polyprotein consisting of the 50-kDa (432 amino acids) protease and the coat protein (CP) expressed from a modified cDNA fragment of the PVY genomic RNA on pPVY2 in E. coli has a self-processing activity, by which the mature CP is released from the polyprotein, although the N-terminal 62 amino acids of the protease were truncated by the internal initiation of the translation. However, introduction of a 14-amino acid deletion overlapping the putative active site residue, Cys339, of the PVY protease (pPVY4) abolished the proteolytic activity. From the studies on the TEV Nla protease, it has been reported that its proteolytic activity is mapped on its C-terminal half.5,6 On the basis of these aspects, we supposed that the N-terminal half of the PVY protease could be also dispensable for its activity.

To examine the moiety responsible for the proteolytic activity within the PVY protease, a series of N-terminally truncated mutants of the protease was constructed by modifying pPVY2 (see Fig. 1). The PVY proteases expressed from these plasmids formed inclusion bodies in E. coli. The activity of each protease was investigated by Western blotting analysis of the inclusion bodies using the anti-PVY CP antibody. The 32-kDa mature CP should be detected while the proteolytic activity remains, like the protein expressed from pPVY2 (Fig. 2, lane 1). If the protease has lost its activity, a polyprotein with larger molecular mass should be detected, like the analysis of the protein expressed from pPVY43 (data not shown). As shown in Fig. 2 (lanes 2–4), the mature CP was detected when the products expressed from pPVY2-171, pPVY2-193, and pPVY2-198 were analyzed, indicating that the truncation of N-terminal region up to 197th amino acid did not affect the proteolytic activity. However, the mature CP disappeared and larger polyproteins were detected when a further 3-amino acid truncation was done (pPVY2-201; Fig. 2, lane 5).
suggests that the truncation of N-terminal 200 amino acids abolished the self-processing activity. The protease lacking the N-terminal 230 amino acids (pPVY2-231) also lost its activity. In Fig. 2, bands of approximately 65 kDa were detected nonspecifically in some lanes.

Dougherty and Parks showed that the 49-kDa Nla protease of TEV has an internal cleavage site between glutamic acid and glycine residues at the amino acid positions 189 and 190. They proposed that the cleavage at this site resulted in the separation of the 49-kDa protein into two domains: a 21-kDa VPg domain and a 27-kDa protease domain that had a proteolytic activity similar to the 49-kDa-associated activity. In the 50-kDa PVY protease, a potential internal cleavage site (Glu/Ala) is also conserved at the amino acid positions 189–190. Our results indicated that the N-terminal 197 amino acids were not required for the proteolytic activity of the PVY protease. Even if the 50-kDa protease is processed at this internal cleavage site, the C-terminal half would be able to act as a protease.

The PVY protease cleaves the polyprotein by an intermolecular cleavage reaction in E. coli

We demonstrated the cleavage of the PVY polyprotein in the situation that the PVY protease and the cleavage site (polymerase-CP junction) were on the same molecule. In this system, both cis (intramolecular) and trans (intermolecular) cleavages could occur. Here, we direct our notice to the trans cleavage activity of the PVY protease. To examine the trans cleavage, we used a binary E. coli expression system, in which the protease and a substrate protein were expressed simultaneously. As the protease producer, pPVY3 was used, which is a high copy number plasmid. The 85-kDa and 34-kDa polyproteins encoded by pPVY4-pAC and pPVY6-pAC, respectively (Fig. 3, lanes 2 and 3), that are medium copy number plasmids and compatible with pPVY3 in E. coli, were used as the substrate proteins, both of which contain the polymerase-CP junction. The proteins expressed from pPVY4-pAC, pPVY6-pAC, and pPVY3 formed inclusion bodies in E. coli cells. When we incubated the inclusion bodies of the substrate proteins overnight together with that of the protease, we could not detect the release of the mature CP (data not shown). On the other hand, when pPVY3 was introduced into E. coli cells harboring pPVY4-pAC or pPVY6-pAC, the 32-kDa mature CP was detected (Fig. 3, lanes 4 and 5), indicating that a rapid trans cleavage occurred in E. coli. In the case of the TEV and plum pox virus (PPV) Nla proteases, each protease cleaves the polymerase-CP junction very efficiently in trans. Some uncleaved molecules remained in our case. Efficient cleavage may be prevented by the aggregation of the protease and substrate molecules in the inclusion bodies.

Next, we examined the requirement of the C-terminal sequence of the PVY protease for its proteolytic activity. The protease expressed from pPVY3 has the complete C-terminal sequence of the wild type protease though it lacks the N-terminal 62 amino acids because of the internal initiation of translation in E. coli (data not shown). Garcia et al. showed that the C-terminal approximately 30 amino acids of the PPV Nla protease can be replaced by nonspecific sequences. We constructed pPVY3-C10 and pPVY3-C33 coding for the mutant proteases; the C-terminal 9 amino acids of the PVY protease were replaced by 3 amino acids (Lys-Leu-Thr) and the 32 amino acids were replaced by 2 amino acids (Leu-Ser), respectively. As shown in Fig. 4A

Fig. 3. Analysis of Intermolecular Cleavage Activities of PVY Protease in E. coli.

An autoradiogram of Western analysis with anti-PVY coat protein antibody is shown. Insoluble fractions from the cell lysate of E. coli cells harboring pPVY2 (lane 1), pPVY4-pAC (lane 2), pPVY6-pAC (lane 3), pPVY4-pAC and pPVY3 (lane 4), pPVY6-pAC and pPVY3 (lane 5), and pPVY3 (lane 6) were electrophoresed. The position of CP is indicated by an arrow on the left side and the mobilities of molecular mass markers are indicated on the right side.

Fig. 4. Analysis of Proteolytic Activities of C-Terminus-truncated Proteases.

An autoradiogram of Western analysis with anti-PVY coat protein antibody and a Coomassie brilliant blue-stained gel are shown in panel A and B, respectively. In both panels, insoluble fractions from the cell lysate of E. coli cells harboring pPVY6-pAC and pPVY3 (lane 1), pPVY6-pAC and pPVY3-C10 (lane 2), pPVY6-pAC and pPVY3-C33 (lane 3), pPVY6-pAC and pPVY3-17C10 (lane 4), and pPVY6-pAC and pPVY3-17C10 (lane 5) were electrophoresed. The position of CP is indicated by an arrow on the left side and the mobilities of molecular mass markers are indicated on the right side. Each truncated protease is marked with an arrowhead.
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Fig. 5. Analysis of Proteolytic Activities of Mutant PVY Proteases.

An autoradiogram of Western analysis with anti-PVY coat protein antibody is shown. Insoluble fractions from the cell lysate of *E. coli* cells harboring pPVY2 (land 1), pPVY4 (land 2), pPVY2-H233Y (land 3), pPVY2-H234Y (land 4), pPVY2-H249Y (lane 5), pPVY2-H238Y (lane 6), pPVY2-D269N (lane 7), pPVY2-D278N (lane 8), pPVY2-C298G (lane 9), pPVY2-C339S (lane 10), pPVY2-C339G (lane 11), and pPVY2-C350G (lane 12) were electrophoresed. The position of CP is indicated by an arrow on the left side and the mobilities of molecular mass markers are indicated on the right side.

(lanes 2 and 3), the release of mature CP from the pPVY6-pAC-encoded 34-kDa substrate protein by the trans cleavage was observed in the *E. coli* cells harboring pPVY3-C10 but not in the cells harboring pPVY3-C33. These results indicated that the replacement of the C-terminal 32 amino acids abolished the proteolytic activity though the replacement of 9 amino acids did not affect the activity. The presence of the mutant proteases was confirmed by Western blotting using anti-PVY protease antibody (data not shown). The 32-amino acid replacement resulted in the deletion of Gly401, the counterpart of which in the PPV Nia protease is Gly406, which is essential for its proteolytic activity.1,4 Our results are consistent with those reported so far.

The truncation of both N-terminal 170 and C-terminal 9 amino acids of the PVY protease (pPVY3-171C10) and both N-terminal 197 and C-terminal 9 amino acids (pPVY3-198C10) did not affect the proteolytic activity, which was concluded from the presence of the mature CP excised from the 34-kDa protein (Fig. 4A, lanes 4 and 5). Based on these results, we concluded that the protease moiety in the 50-kDa PVY protease is the 225-amino acid sequence from Asp198 to Asp422, 26 kDa in size (Fig. 4B, lane 5). The binding activity of our anti-PVY protease antibody to the truncated protease decreased according to the length of the N-terminal truncation and the 26-kDa protease moiety was not recognized with the antibody (data not shown).

The catalytic triad of the PVY protease is composed of His233, Asp269, and Cys339

Bazan and Fletterick10 proposed that the PVY protease is related to the trypsin-like serine proteases and thought that its catalytic triad is composed of His234, Asp269, and Cys339. To verify this, we mutated these amino acid residues in the protease region of PVY polyprotein encoded by pPVY2 by site-directed mutagenesis (pPVY2-H234Y, -D269N, and -C339G) and tested their proteolytic activities by evaluating the amount of the mature CP released from them with Western blotting analysis. The histidine, aspartic acid, and cysteine residues flanking the predicted active sites were also mutated (pPVY2-H233Y, -H249Y, -H258Y, -D278N, -C298G, and -C350G). As shown in Fig. 5, the mature CP was observed in *E. coli* cells expressing the proteases with a mutation at His233, His249, His258, Asp278, Cys298, and Cys350 (lanes 3, 5, 6, 8, 9, and 12), indicating that these amino acid residues are not essential for the self-processing activity. It is noticeable that the replacement of His233 by a bulky tyrosine residue did not abolish the proteolytic activity. On the other hand, replacing His243 with tyrosine abolished the proteolytic activity (Fig. 5, lane 4). By comparison of these results, we conclude that His243 is essential for the proteolytic activity and that His233 can not complement the function of His243 despite its location close to His234. Replacement of Asp269 with asparagine and that of Cys339 with glycine also resulted in the synthesis of polyproteins which were no longer able to self-process (Fig. 5, lanes 7 and 11). On the basis of these results, it is concluded that the catalytic triad of the PVY protease is composed of His234, Asp269, and Cys339, supporting the prediction of Bazan and Fletterick.10 The substitution of His234, Asp269, and Cys339 in the TEV Nia protease and that of His239 in the PPV protease, the counterparts of the active site residues of the PVY protease, also resulted in the loss of the proteolytic activity.9,13 Interestingly, replacement of Cys339 with serine (pPVY2-C339S) did not abolish the self-processing activity and reduced accumulation of the mature CP was observed (Fig. 5, lane 10). This result suggests that the serine residue can function with a low efficiency as the nucleophile in the PVY protease. A similar result has been reported concerning the substitution of Cys339 of the TEV Nia protease.30 In the case of serine protease, Higaki et al.15,17 reported that trypsin retains some proteolytic activity after the active site serine residue (Ser195) is replaced with cysteine. Hahn and Strauss18 also reported that substituting cysteine for Ser215, which is an active site of Sindbis virus capsid protein autoprotease related to the chymotrypsin-like serine protease,19 did not inhibit its proteolytic activity. These results strongly support the relationship between the viral cysteine and trypsin-like serine proteases. Possibly, serine and cysteine residues can function interchangeably as the nucleophile during proteolysis.

References