The Pro-Form of *Stereum purpureum* Endopolygalacturonase I Is Inactivated by a Pro-Sequence in the C-Terminal Region

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The Pro-form (Pro-EndoPG I) of *Stereum purpureum* endopolygalacturonase I has a unique C-terminal region (pro-sequence) that is lacking in PGs of other origins. Mature EndoPG I purified from the culture filtrate of this fungus does not have the 44-amino-acid pro-sequence present in Pro-EndoPG I. We expressed Pro-EndoPG I in *Escherichia coli* and examined its activity. It was found that Pro-EndoPG I had no PG activity, but that PG activity was acquired after the degradation of part of the pro-sequence with V8 protease. These results suggest that the pro-sequence inactivates auto-PG activity. No similar sequence has been reported for any glycoside hydrolase. We then constructed EndoPG I mutants and identified two Glu residues, E364 and E366, that were related to auto-inactivation. A test involving injection of the enzyme into apple trees showed that Pro-EndoPG I induced the same silver-leaf symptoms as mature EndoPG I.

**Key words:** auto-inactivation; pro-sequence; endopolygalacturonase; *Stereum purpureum*; silver-leaf symptom

*Stereum purpureum* endopolygalacturonase I (EndoPG I) is a unique enzyme that has been identified as the substance responsible for the symptoms of silver-leaf disease in apples caused by *S. purpureum*, a pathogenic fungus, and which produces the same symptoms as those in trees naturally infected with the fungus.1-3 This enzyme was originally isolated from a culture filtrate of the fungus (ASP-4B isolate) on the basis of tests involving injection into young apple trees.2 The ASP-4B isolate produced four kinds of enzyme: EndoPG I, II, III, and IV, all having different isoelectric points.4 EndoPG I, which has the highest pI (pH 8.5) of the four, has been studied in detail because it is the major component, accounting for over 50% of the EndoPGs in the isolate, and is also a thermostable PG.3,5,6 The enzymatic and physicochemical properties, and also the X-ray 3D crystal structure at ultra-high resolution (0.96 A), have been clarified.7,8 The primary structure of EndoPG I, deduced by cloning, is 44 amino acid residues longer than mature EndoPG I isolated from the culture filtrate.9,10 These 44 amino acid residues are located at the C-terminal end of EndoPG I. This sequence is lacking in EndoPG IV from the same isolate (accession no. AB252456).11 Although the mature EndoPG I purified from the culture filtrate is approximately 43% homologous with other fungal endoPGs, there is no homology for the 44 residues lacking in the C-terminal region,12-14 although there is high C-terminal homology in the primary structures of EPGB1 and EPGB2 from *Chondrostereum (Stereum)* purpureum isolate 2128u deduced from the respective genes.15 Therefore, this C-terminal sequence appears to play a very important role in the production of silver-leaf symptoms due to EndoPG. In addition, no endoPGs of other origin have been reported to have a pro-sequence resembling the 44 residues in EndoPG I from *S. purpureum*. We have designated the enzyme possessing the 44-residue C-terminal sequence Pro-EndoPG I to distinguish it from mature (active) EndoPG I. It is thought that Pro-EndoPG I, produced by this fungus, is secreted to the external mycelium and then converted to the mature type through the action of proteases secreted in a medium.

In this study, to clarify the function of the pro-sequence, we attempted to express Pro-EndoPG I in *Escherichia coli*. We found that the pro-sequence of Pro-EndoPG I inactivated the PG activity of the enzyme. Moreover, we carried out expression experiments using C-terminal deletion mutants and attempted to identify the amino acid residues related to enzyme inactivation.

**Materials and Methods**

*Isolate, plasmids, culturing conditions, and primers. Escherichia coli* JM109, was used as the host for plasmid propagation. The isolate was cultured in LB medium containing 50 μg/ml of ampicillin. *E. coli*, Origami (DE3) (Novagen, Madison, WI, USA), was used as host for the expression of Pro-EndoPG I, and was grown in LB medium. In this study, Origami was transformed with pT-groE, and co-expressed with chaperone protein GroEL and GroES.16 The plasmid, pT-groE, was kindly provided by Dr. Takashi Yoshida of Hirosaki University. For protein expression in *E. coli*, pET11b vector (Novagen) was selected. All the synthetic oligonucleotides used in this study were obtained from Hokkaido System Science (Sapporo, Japan).

**Construction of an expression plasmid for Pro-EndoPG I and C-terminal deletion mutants.** The expression vector (pET11b/ProPGI) was constructed as follows: The Pro-EndoPG I cDNA was amplified by

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PCR using oligonucleotide primers 5'-GGGAATTCCATATGCTAGTAGTGCCCGGAAGTAGTGGAGTT-3' and 5'-ATAGGATCTCTAGTATGATGATGTTCCCGAAGATGCTGAGT-3' (BamH I). The primers used in PCR introduced Nde I and BamH I restriction sites and a 6 × histidine tag at the 3'-end. About 1,200 bp of PCR fragment was digested with Nde I and BamH I and then ligated to the corresponding sites of pET11b. Then the ligation product was transformed into competent cells of E. coli, JM109. The DNA sequence of the insert was analyzed using an ABI PRISM 3100 sequencer (Applied Biosystems, CA, USA). The procedures for small-scale preparation of plasmid, digestion with restriction enzymes, ligation, and transformation all followed standard methods.

Expression vectors for deletion mutants CT38, CT34, CT32, and CT29 were constructed by the same method as in the construction of pET11b/ProPGI. The same forward primer was used to amplify the inserted DNA. The reverse primers used in the construction of these four mutants are described in Supplemental Table 1 (see Biosci. Biotechnol. Biochem. Web site). Furthermore, the expression vectors for CT31, CT30, and CT28 were constructed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with pET11b/CT32 plasmid as template. The oligonucleotide primers for these mutants are also described in Supplemental Table 1.

Expression of Pro-EndoPG I and other mutants. The E. coli host strains Origami were transformed with expression vectors. Transformants were selected on LB plates supplemented with 50 μg/ml of ampicillin and 34 μg/ml chloramphenicol. A well-grown single colony selected and cultured as described previously. After cultivation, the cell pellets were collected by centrifugation. The cell pellets were resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The suspension was sonicated 3 times for 2 min each on ice with a Branson Sonifier 250 (Branson, Danbury, CT, USA), and was then centrifuged at 20,000 × g for 10 min at 4°C to separate the soluble and insoluble fractions. The PG activity of the soluble fraction was measured.

EndoPG activity and protein assay. EndoPG activity was determined as described previously. The protein concentration was determined by Bradford assay using a BSA standard.

Purification of His-tagged fusion recombinants. The crude extract was applied to a Ni-NTA column (8 mm i.d. × 15 mm, Qiagen, Venlo, Netherlands) equilibrated with a lysis buffer. The column was washed with 5 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the recombinant Pro-EndoPG I was eluted with 4 ml of elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fractions of 250 mM imidazole were pooled and dialyzed. The dialysate was lyophilized and stored at −20°C.

SDS-polyacrylamide gel electrophoresis and Western blotting. SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli. For Western blotting, S. purpureum EndoPG I polyclonal antibody raised in rabbits and anti-His-tag monoclonal antibody raised in mice (Roche, Basel, Switzerland) were used for the primary antibody. Alkaline phosphatase Anti-Rabbit IgG (H+L) (Vector, Burlingame, CA, USA) and alkaline phosphatase Anti-Mouse IgG (H+L) (Vector) were used as the secondary antibody. The protein was visualized using BCIP/NBT alkaline phosphatase substrate Kit IV (Vector).

Activation examination with proteases. V8 protease and carboxypeptidase Y were used in this examination. Fifty μg of purified Pro-EndoPG I was dissolved in 50 mM Tris–HCl buffer (pH 7.8), and 4 units of V8 protease was added to this solution and the reaction mixture was incubated at 25°C. PG activity was examined after 5 h. Likewise, 50 μg of purified Pro-EndoPG I was treated with carboxypeptidase Y in 100 mM acetate buffer (pH 5.0) for 5 h at 30°C, and PG activity was examined.

MALDI-TOF–MS analysis. MALDI-TOF–MS (matrix assisted laser desorption ionization time of flight mass spectrometry) analysis was performed in positive ion mode on a VoyagerTM Workstation (PerSeptive Biosystems, Framingham, MA, USA). Sinapinic acid (5 mg/ml) in 50% CH3CN/0.1% TFA was used as the matrix.

Injection test with young apple trees. The method of Brooks was employed with a slight modification. A 1 ml solution containing 50 μg of purified Pro-EndoPG I was introduced into healthy young apple trees (2 years old) through a glass tube, which was tightly inserted into the hole (5 mm diameter) bored so as to reach the vessel in the main trunk. After the injection of Pro-EndoPG I, the apple trees were observed for 2 weeks in a greenhouse.

Results and Discussion

Expression, purification, and analysis of Pro-EndoPG I

cDNA cloning of EndoPG I has shown that the mature enzyme lacks 44 residues in the C-terminal region that are present in the Pro-form. In the present experiment, Pro-EndoPG I including the C-terminal 44 residues was expressed in E. coli as host. The PG activity in the crude extract from E. coli was 0.25 U/ml as converted to 1 ml of the E. coli culture. This activity was far lower than when mature EndoPG I was expressed by E. coli, at approximately 7%. The maximum activity of the mature-type enzyme was 3.7 U/ml as converted to 1 ml of E. coli culture. His-tagged Pro-EndoPG I was purified to homogeneity from the crude extract using a Ni-affinity column (Fig. 1A), and its molecular weight was clearly higher than that of the recombinant mature EndoPG I (not His-tagged) produced by E. coli. This purified Pro-EndoPG I had no detectable PG activity. The purified protein was also identified by Western blotting with anti-His-tag or anti-PG antibody (Fig. 1B and C). This confirmed that the recombinant enzyme was Pro-EndoPG I possessing 44 more amino acid residues (pro-sequence). These results suggest that Pro-EndoPG I was inactivated by the 44 C-terminal amino acid residues.

However, PG activity, although weak, was detected in the crude extract. Hence we carried out Western blot analyses of the crude extract using two kinds of antibodies (Fig. 2A and B). One band, with a lower molecular weight than His-tagged Pro-EndoPG I, was detected only when anti-PG antibody was used.
purified from the culture filtrate of S. purpureum was 380 U/mg, 15% lower than that of mature EndoPG I. The specific activity of a sample digested with the protease EndoPG I was confirmed to have PG activity. The proteolysis appeared to be limited in extent, since only one band was detected by Western blotting (Fig. 2B).

**Activation of Pro-EndoPG I with proteases**

The above results suggest that Pro-EndoPG I, which has no PG activity, becomes activated upon digestion of the C-terminal region with proteases. Hence we attempted to activate Pro-EndoPG I using two kinds of commercially available proteases. One of the proteases employed was V8 protease, because it includes 13 acidic amino acid residues in the pro-sequence, composed of 44 amino acids. The other was carboxypeptidase Y, which hydrolyzes amino acids one by one from the C-terminus and has wide specificity.

After V8 protease treatment, the digested Pro-EndoPG I was confirmed to have PG activity. The specific activity of a sample digested with the protease was 380 U/mg, 15% lower than that of mature EndoPG I purified from the culture filtrate of S. purpureum. The reason for the lower specific activity is unclear, but the proportion of Pro-EndoPG I molecules subjected to enzymic cleavage might be insufficient to confer full activity. A sample treated with V8 protease was analyzed by SDS–PAGE and Western blotting (Fig. 3A, B, and C), and this revealed that Pro-EndoPG I yielded two bands, with a tendency for the low-molecular-weight form to predominate on SDS–PAGE (Fig. 3A). These two proteins were not detected on Western blotting with anti-His antibody (Fig. 3B). Thus, their C-terminal ends appeared to have been removed. Furthermore, MALDI-TOF-MS analyses of Pro-EndoPG I after V8 protease digestion demonstrated two peaks with mass-to-charge ratios (m/z) of 35,787 and 37,770 for the two proteins, corresponding to the molecular weights that would be expected if cleavages occurred around the E346 and E364 positions (data not shown). These cleavages would result in deletion of 33 amino acid residues needed for auto-inactivation.

On the other hand, because no decrease in molecular weight after carboxypeptidase Y treatment was confirmed by SDS–PAGE, and in addition no PG activity was detected, our results indicate that carboxypeptidase Y does not act on the C-terminus of Pro-EndoPG I. However, Western blot analysis with anti-His-tag antibody showed that only the His-tag was cleaved with the enzyme (data not shown).

Although the ratio of two Pro-EndoPG I digested with V8 protease was unclear, judging from the specific activity (85% of the mature type) and the concentration of the two bands on SDS–PAGE of the digested sample, the possibility that both had PG activity was considered. The reason the specific activity was lower than the native EndoPG I was the difference in the specific activities of the two digested Pro-EndoPG I. It is thought that the specific activity of Pro-EndoPG I having shorter deletion was markedly low. The shorter deletion of the two digested products deleted 15 amino acid residues. Hence, to clarify the number and the important residues of the amino acids on C-terminal side of the Pro-EndoPG I, which are needed for auto-inactivation, we tried to adjust the deletion mutants to about 15 C-terminal residues experimentally and then examined each specific activity.

Although the presence of the pro-type enzyme in the fungal culture medium was confirmed by Western blotting, no purified preparation was obtained. It is difficult to purify the pro-type, because the pro-sequence is easily degraded by proteases secreted into the medium, converting the pro-type to mature EndoPG I.

**Identification of the importance and number of amino acid residues needed for auto-inactivation**

To elucidate the amino acid residues that are important for auto-inactivation, we constructed several C-terminal deletion mutants and analyzed the recombinants. Based on the results of MALDI-TOF-MS analysis of Pro-EndoPG I digested with V8 protease, we expected that some of the C-terminal 15 residues (E364–G379) would participate in auto-inactivation. On the basis of our prediction, seven C-terminal deletion mutants, CT38, CT34, CT32, CT31, CT30, CT29, and CT28, were constructed (Fig. 4A and B).
Each mutant Pro-EndoPG I was produced by *E. coli* and purified by Ni column chromatography by the same method as that for Pro-EndoPG I. We found that CT38, CT34, and CT32 had no PG activity. On the other hand, PG activity was confirmed in CT31, CT30, CT29, and CT28. The specific activities of these mutants are summarized in Table 1. In CT30, activity was unequivocal, and the specific activity was similar to that of CT29. However, the activity of CT28 was markedly increased. These findings clearly indicate that two Glu residues, E364 and E366, are important for auto-inactivation. These data also suggest that 31 or 32 residues of the 44 amino acid residues in the pro-sequence were needed for auto-inactivation. The specific activity of CT28, 155 U/mg, was approximately 60% lower than that of native mature EndoPG I. Although E364 and E366 were important for auto-inactivation, other factors among the remaining amino acids residues constituting the pro-sequence also appeared to play important roles.

**Induction of silver-leaf symptoms with Pro-EndoPG I**

Mature EndoPG I purified from the culture filtrate of *S. purpureum* as a silver-leaf-inducing substance reproduces the same symptoms when injected into young apple trees. Hence we tested the silver-leaf-inducing ability of Pro-EndoPG I by injecting it into apple trees. Two weeks after injection, silver-leaf symptoms were confirmed, being especially conspicuous in the upper leaves (Fig. 5). Since PG activity is needed to induce silver-leaf disease, it is thought that Pro-EndoPG I is digested in the C-terminal region by certain proteases present in apple trees, and thus activated. These proteases might be present in the apoplasm of apple leaves.

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