Characterization of the effects of C-terminal pro-sequence on self-inactivation of Stereum purpureum endopolygalacturonase I

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One sentence summary: This paper describes the analysis of an unknown suppression mechanism of endopolygalacturonase activity using detailed enzyme characterizations and site-directed mutagenesis.

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ABSTRACT

Endopolygalacturonase I from Stereum purpureum has been identified as a causative substance for the silver-leaf disease in apples. It possesses a unique pro-sequence in the C-terminal region that lacks endopolygalacturonases from any other origin. In this study, we analyzed and compared enzymatic characteristics between pro-form (pro-endoPG I) and mature form processed by V8 protease (endoPG I) and described the suppression activity of the pro-sequence. Of note, the optimal pH for pro-endoPG I activity shifted to pH 4.0 from pH 4.5–5.0 of endoPG I. The kinetic parameters indicated that the activity inhibition resulted from a pH-independent decrease of substrate affinity and pH-dependent deterioration of velocity by the pro-sequence. Analysis of site-directed mutations within pro-endoPG I showed that its α-helical structure includes two glutamates (E364 and E366) and alanine (A365), and its orientation by prolines (especially P348) in the pro-sequence played a significant role in its suppression activity. As for mutations in the mature domain, a marked reduction of suppression was observed for enzymes with mutations in H150, R220 and K253, indicating that the pro-sequence interacts with the active cleft by a few ionic bonds.

Keywords: endopolygalacturonase; Stereum purpureum; pro-sequence; inactivation; site-direct mutagenesis; pectin; kinetics

INTRODUCTION

Pectin predominantly consists of partially methyl-esterified α-1,4-linked D-galacturonic acid and is widely found in the middle plant lamellae (Kashyap et al. 2001). Pectin is naturally degraded by pectinase, including pectin methyl esterase (EC3.1.1.11), pectin lyase (EC4.2.2.10), exopolygalacturonase (EC3.2.1.67) and endopolygalacturonase [endoPG, EC3.2.1.15] (Nikolic and Mojonic 2007; Wei et al. 2010). These pectinolytic enzymes from bacteria and fungus play an important role in plant infections (Collmer and Keen 1986). The pathogenic fungus Stereum purpureum endoPG has been identified as the causative substance for the silver-leaf disease in apples; this enzyme produces the same symptoms as those in trees naturally infected with the fungus (Miyairi et al. 1977; Miyairi, Okuno and Sawai 1985). The ASP-4B isolate used in this study produced four forms of the enzyme (endoPG, I–IV) with different isoelectric points (Miyairi and Okuno 1990). The primary structure of endoPG I, deduced by cloning (accession no. AB232992; Miyairi et al. 1997), is 44 amino acid residues longer than that of the mature endoPG I.
isolated from the culture filtrate. These 44 amino acid residues are located in the C-terminal region of endoPG I (Shimizu et al. 2000). A similar sequence is not present in endoPG II, III and IV (accession no. EndoPG II, AB549214; III, AB549215; IV, AB252456) from the same isolate (Ogawa et al. 2009).

So far, although the endoPG gene has been successfully cloned from several bacterial and fungal species, such as Erwinia sp., Pseudomonas sp., Bacillus sp. and Aspergillus sp. (Huang, Li and Wang 2006), the unique 44 amino acid residues of the C-terminal region are not homologous with any other PGs. Therefore, this distinct sequence appears to play a very important role in the production of silver-leaf symptoms. In this study, we have designated the enzyme possessing the C-terminal 44 amino acid region as pro-endoPG I to distinguish it from endoPG I. Although it was previously reported that pro-endoPG I activity was suppressed by the pro-sequence (Ogawa et al. 2010), the suppression mechanism is still unclear. Here, we examined different enzymatic characteristics of pro-endoPG I and compared them with those of endoPG I. Moreover, we analyzed the effect of site-specific mutagenesis of the pro-sequence and its mature domain on its suppression activity, which was also investigated by identifying important amino acid residues that participate in the suppression of enzyme activity.

**MATERIALS AND METHODS**

**Plasmid construction**

*Escherichia coli* XL1-blue was used as a host for plasmid propagation and sequencing. Construction of the expression vector (pET11b/pro-endoPG I) was described previously (Ogawa et al. 2010). Briefly, the pro-endoPG I cDNA was amplified by polymerase chain reaction (PCR) with primers introduced *NdeI* and *BamHI* restriction site and a histidine (*His*) tag at the 3′ end. The PCR fragment was digested by *NdeI/BamHI* and inserted into same sites of pET11b vector (Novagen, Madison, WI, USA). Amino acid replacements were performed using a QuikChange site-direct mutagenesis kit (Stratagene, La Jolla, CA, USA) with pET11b/pro-endoPG I plasmid as template. PCR was performed using PrimeSTAR HS (Takara Bio, Otsu, Japan). For instance, the primers used for the E364Q mutation were 5′-CCAGATAGCTACTATGCAAGCGTGAAGCGAGGAGGCT-3′ (forward) and 5′-AGCCCTTCTTGCCCTACGGGCTTGATGACGATATC TGG-3′ (reverse; underlined sequences indicate mutated codons). Other enzymes with mutations at specific sites were created in a similar way using appropriate primers.

**Expression and purification of pro-endoPG I**

*Escherichia coli* Rosetta-gami 2 (Novagen) was used as a host for the expression of pro-endoPG I and mutant enzymes. The constructed expression vector was transformed into Rosetta-gami 2 using the heat-shock method. The selected transformant was then cultured in lysogeny broth medium supplemented with 50 μg mL⁻¹ of ampicillin and 34 μg mL⁻¹ of chloramphenicol at 37°C with shaking. Isopropyl β-D-1-thiogalactopyranoside was added for induction when the absorbance of the medium at 600 nm was 0.7, and then the cells were grown at 20°C for 25 h. Bacterial cells from 1.2 L cultures were harvested and suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole; pH 8.0). After sonication, extracts were obtained by centrifugation. The supernatant was directly loaded onto a Ni-NTA column (15 × 100 mm; Qiagen, Venlo, the Netherlands) equilibrated with lysis buffer. After the column was washed with lysis buffer, proteins were eluted with a linear gradient of 20–500 mM imidazole in lysis buffer. Active fractions were dialyzed against buffer A (20 mM Tris-HCl buffer, pH 7.5). The dialyzed sample was then applied to a DE52-Cellulose column (15 × 100 mm; Whatman, GE Healthcare Bio-Science, Piscataway, NJ, USA) equilibrated with buffer A. After the column was washed with buffer A, proteins were eluted with a linear gradient of 0–0.5 M NaCl in buffer A. The active fractions were pooled and concentrated with a Vivaspin centrifugal filtration device, with a molecular weight cut-off 10 kDa (Vivascience, Hanover, Germany), and then stored at −20°C after addition of glycerol.

**Protease treatment**

Approximately 10 μg of purified pro-endoPG I was treated with 2 units of V8 protease (Wako, Tokyo, Japan) in 50 mM Tris-HCl buffer (pH 7.8) at 25°C for 16 h; the processed form was designated endoPG I. Both the enzymes endoPG I and pro-endoPG I, treated with the same conditions but without V8 protease, were examined and their enzymatic characteristics were compared.

**Enzyme activity assay**

EndoPG activity was assayed by measuring the amount of reducing sugars released using the method of Milner-Avigad, as described previously (Nozaki et al. 1997). The reaction mixture (final volume, 2.5 mL) consisting of 0.2% polygalacturonic acid sodium salt (Sigma, St. Louis, MO, USA) reaction solution and 100 mM acetate buffer (pH 4.5) was preincubated at 30°C for 5 min. The reaction was initiated by the addition of the enzyme, incubated at 30°C for 10 min and then terminated by the addition of a copper solution, according to the Milner-Avigad method. One unit of PG activity was defined as the quantity of enzyme necessary to release 1 μmol of reducing sugars per minute under the above conditions. When the optimum pH was determined, 100 mM acetate buffer (pH 3.0–5.5) and 80 mM MES buffer (pH 5.5–7.0) were used as a reaction buffer. For pH stability, the enzyme was kept in Brinton–Robinson buffer (diluted 1:5; pH 2.0–12.0) at 4°C for 16 h. Then, PG activity was measured under normal pH conditions with 100 mM acetate buffer (pH 4.5).

To analyze the optimum temperature, purified enzymes were reacted at different temperatures (30–80°C) in the above standard conditions. In the case of thermal stability, the purified proteins were heat-treated at different temperatures (30–75°C) for 30 min, and then immediately cooled on ice to analyze residual activity. The kinetic parameters of pro-endoPG I (at pH 4.0 and 4.5) and endoPG I processed by V8 protease (at pH 4.5) with polygalacturonic acid and pectin were derived from Lineweaver-Burk plots. The Km and kcat values were derived from means of three independent experiments in each substrate concentration. When the effect of site-directed mutagenic enzymes on enzyme activity suppression was measured, the pH of the reaction mixture (pH 5.0) was used to clearly detect any decrease in suppression caused by the mutations. The contribution ratio of the mutations in activity repression was determined as a percentage relative to the specific activity following V8 protease treatment, which was set to 100%. The protein concentration was determined according to the Bradford method (Bradford 1976) using bovine serum albumin as a standard.
SDS-PAGE was performed on a slab gel prepared with a 3% acrylamide stacking gel and 10% acrylamide separating gel, as described previously (Laemmli 1970). Proteins were stained with Coomassie Brilliant Blue. Proteins separated by SDS-PAGE were then transferred onto nitrocellulose membranes. Immunoblot analysis was performed with a primary S. purpureum endoPG I rabbit polyclonal antibody (Senda et al. 2001) and a mouse anti-His6-tag monoclonal antibody (Roche, Basel, Switzerland), as described previously (Hamada et al. 2001). Alkaline phosphatase-conjugated anti-rabbit IgG (H + L) and anti-mouse IgG (H + L; Vector, Burlingame, CA, USA) were used as secondary antibodies. Signals were detected by BCIP/NBT Alkaline Phosphatase Substrate Kit IV (Vector).

RESULTS AND DISCUSSION

Purification of pro-endoPG I and activation by protease

To investigate the enzymatic properties of pro-endoPG I, recombinant protein obtained by expression in E. coli. Pro-endoPG I was purified to almost homogeneity through two chromatographic steps (Table 1); the purified enzyme migrated as a single band on SDS-PAGE (Fig. 1A). Because previous results showed that pro-endoPG I had almost no activity in assay conditions at pH 5.0, purification and characterization of pro-endoPG I was difficult. Here, we found that the activity suppression was pH-dependent and defeasible by changing the pH from 5.0 to 4.5. The final enzyme preparation, purified more than 187-fold, consisted of 0.23 mg of pro-endoPG I with a specific activity of 435 U mg⁻¹ at pH 4.5 in assay conditions compared to that of endoPG I. Moreover, the pH range of endoPG I (Fig. 2) was halved to be around pH 4.0, which was 3.8-fold higher than that of pro-endoPG I. EndoPG I (V8 protease-treated pro-endoPG I) and original pro-endoPG I were used in following enzyme characterization.

Comparison of enzymatic characteristics between pro-endoPG I and endoPG I

To determine the effect of the C-terminal extension in pro-endoPG I on enzyme function, the physical properties of purified pro-endoPG I were measured and compared with those of endoPG I (Fig. 2). Of note, pro-endoPG I was most active at around pH 4.0, and the optimal pH shifted to more acidic conditions compared to that of endoPG I. Moreover, the pH range showing activity also significantly narrowed (Fig. 2A). For these reasons, we failed to notice enzyme activity at pH 5.0 in our previous research. However, pH-dependent activity suppression is
considered to be a crucial behavior for symptoms of silver-leaf disease caused by *S. purpureum* because the pH of plant intercellular space is known to be pH 5.0–5.5. Pro-endoPG is efficiently transported in its inactive form from the infected apple trunk to the leaves, where it is possibly digested by certain proteases in apple leaves. The pH stability range of pro-endoPG I (pH 3.0–7.0) was also smaller than that for endoPG I (pH 2.0–10.0; Fig. 2B). Although pro-endoPG I and endoPG I were most active at approximately 60°C (Fig. 2C), more than 90% of the original activity was retained at temperatures up to 60 and 50°C, respectively, when the enzymes were maintained for 30 min at various temperatures ranging from 30 to 75°C (Fig. 2D).

The kinetic parameters of pro-endoPG I were analyzed with polygalacturonic acid or pectin as a substrate and compared with those obtained for endoPG I. Furthermore, the change of pro-endoPG I parameters with pH alteration was also examined (Table 2). Lineweaver–Burk plots of the reaction catalyzed by pro-endoPG I and endoPG I at pH 4.5 showed that the *Km* values for polygalacturonic acid were 0.5 and 0.16 mg mL$^{-1}$, respectively, and *kcat* values were 334 and 996 s$^{-1}$, respectively. In contrast, *Km* and *kcat* values for pectin were calculated to be 1.81 mg mL$^{-1}$ and 12.4 s$^{-1}$ for pro-endoPG I, respectively, and 0.46 mg mL$^{-1}$ and 230 s$^{-1}$ for endoPG I, respectively. (Table 2 and Fig. S1, Supporting Information). Although both enzymes obviously preferred to react with polygalacturonic acid, the catalytic efficiency (*kcat/Km*) indicated that pectin was an unfavorable substrate for pro-endoPG I. The relative ratio of *kcat/Km* for pro-endoPG I with polygalacturonic acid and pectin was 10.7 and 1.4%, respectively, whereas that for endoPG I was defined as 100% (Table 2). These data indicate that pectin is a major component of the plant cell wall structure, pro-endoPG I exists in its inactive form in vivo due to its substrate specificity and change in optimal pH. These data support above our estimation concerning a physiological function of pro-endoPG I that the inactive form involves in the efficient transport of enzyme from apple trunk to leaf.

Table 2. Kinetic parameters.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>endoPG I (pH4.5)</th>
<th>pro-endoPG I (pH4.5)</th>
<th>pro-endoPG I (pH4.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For polygalacturonic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Km</em> (mg mL$^{-1}$)</td>
<td>0.16</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td><em>kcat</em> (s$^{-1}$)</td>
<td>996</td>
<td>334</td>
<td>1670</td>
</tr>
<tr>
<td><em>kcat/Km</em> [s$^{-1}$/mg mL$^{-1}$]</td>
<td>6225 [100]</td>
<td>668 [10.7]</td>
<td>3340 [53.7]</td>
</tr>
<tr>
<td>For pectin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Km</em> (mg mL$^{-1}$)</td>
<td>0.46</td>
<td>1.81</td>
<td>1.92</td>
</tr>
<tr>
<td><em>kcat</em> (s$^{-1}$)</td>
<td>230</td>
<td>12.4</td>
<td>20.2</td>
</tr>
<tr>
<td><em>kcat/Km</em> [s$^{-1}$/mg mL$^{-1}$]</td>
<td>500 [100]</td>
<td>6.9 [1.4]</td>
<td>10.2 [2.0]</td>
</tr>
</tbody>
</table>

*[]: Relative ratio of catalytic efficiency.*

Figure 2. Comparison of some enzymatic properties between pro-endoPG I (solid circles) and endoPG I (open triangles). Panels (A) and (C) show effects of pH and temperature on the enzymatic activity, respectively. Values are shown as percentages of the optimal activity, considered to be 100% (B) After a 16-h incubation at different pH at 4°C, residual activities were measured to assess pH stability. Each maximum activity (pro-endoPG I, pH 4.0; endoPG I, pH 8.0) corresponds to 100%.

(D) After 30 min of incubation at different temperatures, residual activities were measured to assess thermal stability. Each maximum activity (pro-endoPG I, 30°C; endoPG I, 40°C) corresponds to 100%.
The Lineweaver-Burk plots of the reaction catalyzed by pro-endoPG I at pH 4.0 yielded $K_m$ and $k_{cat}$ values of 0.5 mg mL$^{-1}$ and 1670 s$^{-1}$, respectively, for polygalacturonic acid, and 1.92 mg mL$^{-1}$ and 20.2 s$^{-1}$, respectively, for pectin. Interestingly, $K_m$ values for each substrate were almost the same between pH 4.5 and 4.0 for pro-endoPG I, indicating that the inhibition of substrate binding by the pro-sequence is independent of the pH. On the other hand, comparison of $k_{cat}$ values showed that the pro-sequence largely reduced the reaction velocity in a pH-dependent manner. Below, we further examine and discuss the important amino acids concerning interaction between the pro-sequence and mature domain for activity suppression.

**Analysis of mutant enzymes**

Our previous data on deletion mutants in the pro-sequence showed that at least 31 N-terminal amino acid residues in the 44 amino acid pro-sequence are needed to suppress activity and the deletion mutant possessing 28 N-terminal residues of the pro-sequence immediately lost this suppressive activity (Ogawa et al. 2010). Furthermore, our data already showed that the carboxy peptidase Y could digest only His$_6$ tag from C-terminus of pro-endoPG I, and the pro-endoPG I without His$_6$ tag also maintained self-inactivation. This observation indicated the C-terminal His$_6$ tag has no or little contribution to self-inactivation (Ogawa et al. 2010). Several secondary structure prediction programs predicted that the pro-sequence has $\alpha$-helical structure in the neighborhood of residues 28–31 (Fig. 1B). These results suggest that the $\alpha$-helical structure plays an important role in activity suppression. Therefore, we focused on only three of the acidic amino acid residues (D359, E364 and E366) in the $\alpha$-helix and A365 to break the $\alpha$-helical structure (Fig. 1B). Our previous unpublished data have shown that circular dichroism (CD) was not suitable for discriminating analysis of the structural changes of the $\alpha$-helix by these mutations, because there was not much difference in CD spectrums between original pro-endoPG I and endoPG I. Although each contribution ratio of D359N, E364Q and E366Q showed low values of 11.7, 19.9 and 14.2%, respectively, the double mutant E364Q/E366Q remarkably presented the largest contribution (61.8%) (Fig. 3). These results suggest that these acidic residues act cooperatively to suppress enzyme activity. Interestingly, the A365P mutation also showed a large contribution (70.5%) to activity suppression and suggests that the $\alpha$-helical structure in the pro-sequence is essential for its suppressive activity (Fig. 3). We also introduced mutations into 11 basic amino acid residues of the active cleft in the main domain of pro-endoPG I (Shimizu, Miyairi and Okuno 2002). As a result, we found that the contribution ratios in amino acid residues H150A, R220A and K253A were 24.6, 11.3 and 50.6%, respectively. For the other mutant enzymes, excluding K88A, we only detected a minimal contribution (0.52%), and there were no effects on activity suppression (Fig. 3).

Although it is already known that H150 is one of the substrate-binding residues in the +1 subsite (Shimizu, Miyairi and Okuno 2002), the model structure shows that the other two residues were also located in the neighborhood of same subsite (Fig. 4A–C). In particular, K253 is close to H150 and other substrate-binding residues in the +1 subsite compared to R220,
which corresponds to their contribution ratios for activity suppression. These results suggest that ionic bonds between acidic amino acid residues in the pro-sequence and basic amino acid residues in the main domain largely affect active sites in a pH-dependent manner, resulting in a decreased reaction velocity. This is also consistent with the change of optimal pH in pro-endoPG I to pH 4.0. In general, the proton dissociation constant of $\beta$-COOH in aspartic acid and $\gamma$-COOH in glutamic acid was observed around pH 4.0, corresponding to the optimal pH of pro-endoPG I. In other words, the carboxylic anions interact with above basic amino acids at higher than pH 4.0, which results in activity suppression.

Furthermore, we also introduced mutations into five Pro residues (P339, P348, P352, P355 and P358) thought to be important for the orientation of the pro-sequence because Pro provides a fixed $\phi$ angle in peptide bonds. Moreover, P339A, P348A, P352A, P355A and P358A showed 4.3, 80.9, 36.9, 27.6 and 9.3% contribution ratios, respectively. In particular, P348 is thought to be extremely important for activity suppression by properly orienting the $\alpha$-helical structure. The pH-independent inhibition of substrate binding indicated by the Lineweaver-Burk plots is because of the appropriate arrangement of the $\alpha$-helical structure around the +1 subsite by these Pro (Fig. 4C). To clarify this issue, we are now attempting to determine the 3D structure of pro-endoPG I in its inactive form.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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