Auto-inactivation of *Stereum purpureum* Proendopolygalacturonase I by C-terminal 44 Amino Acid Residues

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Abstract: 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 initially but some was acquired after the degradation of a portion of the pro-sequence with V8 protease. These results suggest that the pro-sequence inactivates auto-PG activity. No similar characteristic has been reported for any glycoside hydrolase. We then constructed C-terminal deletion mutants of Pro-EndoPG I and showed that 31 or 32 residues of the 44 amino acid residues in the pro-sequence were needed for the inactivation. Furthermore, we identified two Glu residues, E364 and E366, that were also related to the 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. It is assumed that the Pro-EndoPG I was activated with plant proteases.

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

*Stereum purpureum* endopolygalacturonase I (EC 3.2.1.15) 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 which produces the same symptoms as those in trees naturally infected with the fungus.¹ ² The silvery appearance is accounted as the irregular reflection of light from the leaf surface in which intercellular air-spaces are made by the partial separation of the epidermal cells from the palisade cells. The characteristic symptom of silver-leaf disease is a development of foliage with a silvery sheen; however, the pathogenic fungus is observed only in the wood and not in the leaves. Therefore, it was believed that a substance produced by the fungus was responsible for the development of silver-leaf symptoms.³ ⁴ The endopolygalacturonase (EndoPG I) that we have isolated and identified hydrolyzes a pectin substance in the middle lamella upon reaching the leaves, resulting in silver-leaf symptoms.¹ ²

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 points⁵ and silver-leaf-inducing ability. 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.⁶ ⁷ EndoPG I has been separated into three components (Ia, Ib and Ic) by CM52 column chromatography in spite of an identical isoelectric point.⁶ The number of N-linked oligosaccharides (mostly M5 high-mannose type) for each EndoPG I has been shown to be two for Ia, three for Ib, and four for Ic.⁶ ⁷ Enzymatic properties were found common between EndoPG I and EndoPG IV are yet unknown. Although amino acid sequence homology of mature EndoPG I with EndoPG IV has also been clarified by analysis of the crystal complex, the reasons for such an absence are yet unknown. Although amino acid sequence homology of mature EndoPG I with EndoPG IV (accession no. AB252456) from the same isolate was 72%, this sequence is lacking in EndoPG IV.¹⁰ The position of three disulfide bonds in EndoPG I has also been clarified based on ESI-MS analysis of peptides from tryptic digestion of the enzyme.⁹ Structural analysis by X-ray crystallography has given results at resolutions up to 0.96 Å.¹⁰ Part of the enzyme’s catalytic mechanism has also been clarified by analysis of the crystal complex (binary and ternary) occurring in reaction between enzyme and substrate.¹⁰

We have designated the enzyme possessing the 44-residue C-terminal sequence as Pro-EndoPG I to distinguish it from mature EndoPG I. It is thought that Pro-
Gibberella fujikuroi osporioides f. sp. lycopersici PG (Q74244), Aspergillus niger PGA (Q9P4 W4), Gibberella fujikuroi PG3 (Q5W375), Pro-EndoPG I (P79074), S. purpureum EPG B (Q9PSM4).

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. Mature EndoPG I with PG activity has also been successfully expressed in Echerichia coli(54) and Pichia pastoris. In this study, to clarify the function of the pro-sequence, we attempted to express Pro-EndoPG I in E. coli and P. pastoris. We found that the pro-sequence of Pro-EndoPG I inactivated the PG activity of the enzyme.

Unique C-terminal region of S. purpureum Pro-EndoPG I.

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 that are lacking in the C-terminal region(15-17) (Fig. 1). However, there is a high C-terminal homology in the primary structures of EPGB1 and EPGB2 from the same fungal Chondrostereum (Stereum) purpureum isolate 2128u deduced from their respective genes. Therefore, this C-terminal sequence of EndoPG I seems to play a very important role in the production of silver-leaf symptoms. In addition, no endoPGs from other origins have been reported to have a pro-sequence resembling the 44 residues in EndoPG I from S. purpureum. C. purpureum isolate 2128u produces five kinds of enzymes: EPGA, EPGB1, EPGB2, EPGC, and EPGD deduced from their respective endoPG genes, epgA, epgB1, epgB2, epgC, and epgD. Among the above mentioned PGs, only two are almost identical (97.1% homology) and have a pro-sequence. Likewise, only one of four kinds of PG from ASP-4B isolate has a pro-sequence.

The pro-sequence, which is composed of 44 amino acid residues, contains 13 acidic amino acid residues. Furthermore, the unique arrangement, -DNPDPEDDP-, observed in this sequence was found in sarcosine oxidase by a homology search of the database. However, its function is still unknown.

Expression and analysis of the Pro-EndoPG I in P. pastoris.

For effective production of the Pro-EndoPG I including the C-terminal 44 residues, we first attempted expressing it in P. pastoris. After ammonium sulfate precipitation of the obtained culture filtrate, the PG activity was confirmed through the DE52 column chromatography fraction.
Expression and Analysis of the Pro-EndoPG I in E. coli

In the present experiment, the Pro-EndoPG I 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.¹⁰ The Pro-EndoPG I with a His-tag at the C-terminus was purified to homogeneity from the crude extract using a Ni²⁺-affinity column (Fig. 3A), and its molecular weight was clearly higher than that of the recombinant mature EndoPG I 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 (Figs. 3B and C). This confirmed that the recombinant enzyme was Pro-EndoPG I, possessing 44 more amino acid residues (pro-sequence). These results suggest that the 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 (Figs. 4A and B). One band, with a lower molecular weight than His-tagged Pro-EndoPG I, was detected only when anti-PG antibody was used (Fig. 4B). The results of Western blot analysis indicated that some of the Pro-EndoPG I is hydrolyzed in part of the C-terminal region by proteases from *E. coli*, and thus is activated. The proteinase appeared to be limited in extent, since only one band was detected by Western blotting (Fig. 4B).

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 the Pro-EndoPG I using two kinds of commercially available proteases. One of the proteases employed was V8 protease,²⁰ because it includes 13 acidic amino acids in the pro-sequence, composed of 44 amino acids. The other was carboxypeptidase Y,²⁰ 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*.²⁰ 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 (Figs. 5A, 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. 5A). These two proteins were not detected on Western blotting with anti-His-tag antibody (Fig. 5B). Thus, their C-terminal ends appeared to have been removed. Furthermore, MALDI-TOF MS analyses of the Pro-EndoPG I after V8 protease digestion demonstrated two peaks with mass-to-charge ratios (m/z) of 35,787 and 35,767.

![Fig. 3. SDS-PAGE and Western blotting of Pro-EndoPG I purified by Ni²⁺-affinity column chromatography.](image)

(A) SDS-PAGE analysis of purified Pro-EndoPG I. Proteins separated in the 12.5% polyacrylamide gel were stained by the silver staining. (B) Western blot analysis with anti-His antibody. A His-tag is added to the C-terminus of the Pro-EndoPG I. (C) Western blot analysis with anti-PG antibody. Lanes: M, molecular weight marker; 1, purified Pro-EndoPG I; 2, recombinant mature EndoPG I (not His-tagged) produced by *E. coli*.²⁰

![Fig. 4. Western blotting of *E. coli* crude extract.](image)

(A) Western blot analysis with anti-His antibody. (B) Western blot analysis with anti-PG antibody. Lanes: 1, crude extract; 2, recombinant mature EndoPG I.

![Fig. 5. SDS-PAGE and Western blotting of Pro-EndoPG I digested with V8 protease.](image)

(A) SDS-PAGE analysis of V8 protease-digested Pro-EndoPG I. (B) Western blot analysis with anti-His antibody. (C) Western blot analysis with anti-PG antibody. Lanes: M, molecular mass markers; 1, purified Pro-EndoPG I; 2, Pro-EndoPG I digested with V8 protease; 3, recombinant mature EndoPG I.
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 and 15 C-terminal amino acid residues respectively. Thus, V8 protease appeared to act at a restricted position on the C-terminal side of acidic amino acids in the pro-sequence. These experimental results demonstrated that the C-terminal region (pro-sequence) of the Pro-EndoPG I was responsible for inactivation of the PG activity.

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 the 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 the two Pro-EndoPG Is 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 for the native mature EndoPG I was the difference in the specific activity of the two Pro-EndoPG Is. It is thought that the specific activity of Pro-EndoPG I having shorter deletion (15 amino acid residues) was markedly low. Hence, to clarify the number and the important residues of the amino acids on the 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 amino acid residues 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 the Pro-EndoPG I digested with V8 protease, we expected that some of the 15 C-terminal 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 (Figs. 6A 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 are needed for auto-inactivation. However, 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. 7). Since PG activity is needed to induce silver-leaf symptoms, 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 apoplast of apple leaves.

What advantage does S. purpureum gain in nature by auto-inactivation of its EndoPG I with the C-terminal region of the molecule? This is a very interesting question to consider when discussing the evolution of virulence in phytopathogenic fungi.
Two weeks after injection, silver (5 mm diameter) bored so as to reach the vessel in the main trunk.

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Fig. 7. Silver-leaf of apple tree induced by the Pro-EndoPG I.

The method of Brooks\(^4\) was employed with a slight modification.\(^2\) A 1 mL solution containing 50 µg of the purified Pro-EndoPG I was introduced into healthy young apple trees (2 years old) through a glass tube, which was tightly inserted into a hole (5 mm diameter) bored so as to reach the vessel in the main trunk. Two weeks after injection, silver-leaf symptoms were confirmed.
銀雅病菌由来プロエンドポリガラクツロナーゼ I の
C 末端 44 残基による自己不活性化
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リング銀雅病菌 (Streptococcus ASP-4B) 由来エン
ドポリガラクツロナーゼ I (EndoPG I) のプロ体は他源
の PG にはみられないユニー-な C 末端領域 (プロ配列)
を有している。この配列は本菌の培養液から精製され
た成熟型 EndoPG I にはみられない、本研究ではこのプロ
体を大腸菌に発現させ、それらの性状を解析した。その
結果プロ体には活性がみられなかったが、V8 プロテアーゼ
を用いプロ配列の一部を消化したものは活性が現れた。
これらの結果はプロ配列が自己の不活性化に関与してい
ることを示唆している。これまで報告されたβ糖質加水分
解酵素で、このような性質を有するものは、この不活
性化にプロ配列 44 残基のうち必要とするアミノ酸残基
数、また重要なアミノ酸を明らかにするために C 末端領域
の欠失変異体を作成し、その比活性を測定した。その結果
プロ配列の 31 から 32 残基が必要であり、364 番目と 366
番目のグルタミン酸が特に重要であることが示されたに
になった。プロ体をリングの菌株に注入した結果、成熟型
と同様銀雅症状を呈した。プロ体は、植物由来のプロテ
アーゼにより植物の中で活性化しているものと考えられる。

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【質問】
北大理 - 農 - 松井
切断された C 末端 44 残基のその後の行方は？この残基
が毒素と関連されたことではなくなかなかんか？

【答】
これまでのリング菌株への注入試験により、毒素の本
体がエンドポリガラクツロナーゼであることを複数の方
法で証明しています。また、成熟型に銀雅発現能がある
ことから切断されたペプチドの方に活性はないと考えて
いています。植物中で分解された C 末端 44 残基の行方は
調べていませんが、植物中では 44 残基でできていなく切
断されるのではなく、最終的に 44 残基が切れ成熟タイプにな
るのではないかと推察しています。

【質問】
江崎グリコ - 高田
大腸菌で発現させた ProPG に活性がないことはクリア
に示されているが、元の菌が生産した ProPG にも活性が
いないことは確かなのでしょうか？

【答】
銀雅病菌が生産した Pro-EndoPG I は培地中に分泌後、
速やかに成熟型に変化すると考えられます。この Pro-
EndoPG I は培養初期の液を用いたウエスタンプロット
で C 末端が一部が分解したものしか検出できませんでした。したがって、銀雅病菌が生産した Pro-EndoPG I
を精製することは難しく活性の有無は調べられない状況
です。

【質問】
食総研 - 名古屋
プロ配列をもってい自己不活性化していることの、
銀雅病菌に対して有利に働く理由は何であるか？

【答】
今回のかん見結果の一番興味深い観点だと思います。こ
の回答を以下のように整理してお答えいたします。
○リングの菌株 (2 年生) を用いた銀雅発現試験で銀
雅発現には PG 活性が必要であることが明らかにされてい
ます。
○実際にリング菌で銀雅を呈する罹病樹は樹齢の高い大
きな木が多く、若木は抵抗力のないか罹病しません。
また、若木は生存できず木質部のみ存在しています。
○銀雅病菌にとって木の葉を銀雅するとは光合成
能の低下を誘発し、木の成長 (生命力) を弱めることにつ
ながります。このことは結果的菌の感染部位を拡大し、
勢力を増大させることになると考えられます。
以上の観点からプロ体で分岐させることのメ
リットは、以下のような理由を考えています。
感染木質部で分岐された PG が目的地である葉にまで到
達するには、いろいろな障害が想定されます。目的地ま
での距離に応じて途中に存在する基質となり得るベクチン
質との出会い、この PG の道草による酵素の劣化も考えら
れます。これらのハードルをクリアし、葉に速やかに到
達して PG 活性を発揮するためには、途中で “道草” をせ
ずに済む不活性化が有効と考えられます。