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Glycosylation Status and Conformational Stability of Recombinant *Stereum purpureum* Endopolygalacturonase IVs Produced in an *Aspergillus oryzae* Expression System

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Shun Ogawa,¹ Mariko Ito,¹ Hajime Ohki,¹ Tsubasa Kimura,¹ Tetsuya Shimizu,²
Motoki Matsuda¹ and Kazuo Miyairi^{1,*}

¹Faculty of Agriculture and Life Science, Hirosaki University
(3 Bunkyo-cho, Hirosaki, Aomori 036-8224, Japan)

²RIKEN, Harima Institute at SPring-8
(1-1-1 Kouto, Sayo-cho, Sayo-gun, Hyogo 679-5148, Japan)

Abstract: *Stereum purpureum* endopolygalacturonase (EndoPG) IV was expressed in *Aspergillus oryzae*. Recombinant forms of EndoPG IV (rEndoPGs IV₀, IV₁ and IV₂) were purified from the culture filtrate by ammonium sulfate precipitation and two-step column chromatography with DE52 and hydroxyapatite. Each of these three recombinants was shown to be homogeneous by SDS-PAGE. Since it was predicted that the differences between them would be based on the number of sugar chains, the changes in molecular mass after enzymatic cleavage of the *N*-linked oligosaccharide with EndoH were determined by MALDI-TOF MS. From the data, the numbers of *N*-linked oligosaccharides were estimated to be 0, 1 and 2 for rEndoPG IV₀, IV₁ and IV₂, respectively. These sugar chains appeared to be a mixture of GlcNAc:Hexoses₅₋₁₀ and to include galactose as well as mannose. Mass analyses of IV₀, IV₁ and IV₂ after deglycosylation with EndoH demonstrated a hexose carbohydrate, suggesting the presence of an *O*-linked oligosaccharide that is lacking in native EndoPG IVs. The thermal denaturation curves of rEndoPG IV₀ and IV₁ based on CD analysis indicated *T*_m values of 60 and 59°C, with a one-step thermal denaturation curve, similar to that of native EndoPG IV.

Key words: endopolygalacturonase, *Stereum purpureum*, *Aspergillus oryzae* expression system, glycosylation, CD analysis

Endopolygalacturonase (EC 3.2.1.15) catalyzes random hydrolysis of the α -1,4-glycosidic linkages in polygalacturonic acid, a component of pectin.¹⁾ Endopolygalacturonase (EndoPG) IV is one of four EndoPGs (EndoPGs I, II, III and IV) with different isoelectric points produced by a *Stereum purpureum* isolate, ASP-4B.²⁾ These EndoPGs have been isolated as a silver leaf-inducing substance^{3,4)} and their amino acid sequences have been revealed by cloning (Accession Nos.: EndoPG I, AB232992; II, AB549214; III, AB549215; IV, AB252456).⁵⁻⁷⁾ Among them, only EndoPG I, which was subsequently renamed pro-EndoPG I, has a pro-sequence consisting of 44 amino acid residues,⁸⁾ and shows no enzymatic activity. It is a unique pro-enzyme that appears to be converted to its active form in culture by cleavage of the C-terminal region.⁹⁾ The deduced primary structures of mature EndoPG I and IV obtained from the culture medium show 72% homology, and retain three disulfide bonds.⁷⁾ Furthermore, an expression system has been constructed using *Escherichia coli* Origami as a host.^{7,10)} The specific activity of the recombinant form of mature EndoPG I was similar to that of the native type. However, the thermal denaturation curve for the recombinant obtained by circular

dichroism (CD) spectrum analysis showed two steps in the sigmoid curve, suggesting a mixture of two components with different *T*_m values. Moreover, these two components were found to be the complete (having three disulfide bonds) and incomplete enzymes by electrospray ionization mass spectrometry (ESI-MS) analysis of EndoPG I digested with trypsin. The component with the lower *T*_m value was the incomplete form of EndoPG lacking either the second or third disulfide bond. The proportion of the incomplete enzyme was about 40%, as shown in the thermal denaturation curve. We also obtained similar data using a recombinant expression system in *Pichia pastoris*.¹¹⁾ Therefore, to express the perfect fungus should be used as a host.

The fungal genus *Aspergillus* has been widely used for development of enzyme expression systems. Systems for expression of *Penicillium* sp. glucanase,¹²⁾ *Myceliophthora thermophila* laccase¹³⁾ and *Thielavia terrestris* glucoamylase¹⁴⁾ in *A. oryzae* have been reported. Moreover, Woosley *et al.*¹⁵⁾ have performed a detailed analysis of a comprehensive glycan recombinant of endoPG C expressed in *A. niger*. They established and clarified the structure of *O*-linked and *N*-linked carbohydrate chains and their binding sites. It was expected that carbohydrate chain modifications would occur in recombinants when *Aspergillus* sp. was used as a host. In fact, the recombinant of *Penicillium* exo- β -glucanase

*Corresponding author (Tel. & Fax. +81-172-39-3772; E-mail: km124@cc.hirosaki-u.ac.jp).

expressed in *A. oryzae* was modified by *N*-glycosylation.¹²⁾ It was clarified that in the case of *M. thermophila* laccase, the carbohydrate chains increased the molecular weight by 40–60%.¹⁶⁾ *A. niger* isopullulanase (IPU) expressed in *A. oryzae* showed a molecular mass increase of 20 kDa as a result of carbohydrate chain modification, in comparison with that of the native type.¹⁷⁾ *Erwinia carotovora* subsp. *atroseptica* pectate lyase 3 expressed in *A. niger*, *A. nidulans* and *A. awamori* had been reported to differ in its sugar chains among the three *Aspergillus* types.¹⁸⁾ As described above, although there have been some reports describing the carbohydrate chains of recombinants obtained using a heterologous expression system in *A. oryzae*, no detailed studies have been performed so far.

In the present study, we attempted to obtain a recombinant with a conformational stability based on a complete set of disulfide bonds by using an expression system in *A. oryzae*. It was expected that the recombinant would be modified with carbohydrate chains when this host was used. *S. purpureum* EndoPGs I and IV have four and two *N*-linked carbohydrate chain motifs, respectively, whose structures and positions were already known.^{7,8)} Hence we used EndoPG IV to allow easy analysis of the carbohydrate chains, since it has a small number of carbohydrate chain-binding motifs. This yielded detailed information on the glycosylation of recombinants expressed in *A. oryzae*. Furthermore, on the basis of T_m values obtained from CD thermal denaturation curves, we clarified that the recombinants expressed in *A. oryzae* were similar to native EndoPG IVs in terms of conformational stability, suggesting the presence of a complete set of disulfide bonds, unlike the situation for recombinant EndoPGs (rEndoPGs) of both *E. coli* and *P. pastoris*.

MATERIALS AND METHODS

Strains, plasmids, primers and rEndoPG IV. *E. coli* JM109 was used as the host for plasmid propagation. The strain was cultured in LB medium containing 50 µg/mL ampicillin. *A. oryzae* niaD300 strain was used as the host for expression of EndoPG IV. For protein expression in *A. oryzae*, the pNAN8142 vector was selected. These hosts and the plasmid vector were obtained from Ozeki Co., Ltd. (Nishinomiya, Japan).^{19,20)} All synthetic oligonucleotides used in this study were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). rEndoPG IV expressed in *E. coli* was prepared according to the previous report.¹⁰⁾

Construction of an expression plasmid for EndoPG IV.

The expression vector employed, pNAN8142, has an improved promoter from *A. oryzae*. The pNAN8142/EPGIV expression vector was constructed by amplifying EndoPG IV cDNA by PCR using the oligonucleotide primers 5'-TTTTAAGCTTATGCCTTCCTTCTCTCCCTGA-3' (HindIII) and 5'-TTTGTCTAGATTATGATGTCTCTGAGGAGTCG-3' (Xba I). About 1,200 bp of the PCR fragment, containing the *S. purpureum* signal sequence, was digested with HindIII and XbaI, and then ligated into the corresponding sites of pNAN8142. 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 310 sequencer (Applied Biosystems, Inc., Foster City, USA). The procedures for small-scale preparation of the plasmid,

digestion with restriction enzymes, ligation and transformation, were all performed using standard methods.

Transformation of *A. oryzae* and culture conditions. The constructed expression vector, pNAN8142/EPGIV, was transformed into *A. oryzae* niaD300 strain by the protoplast-PEG method.²¹⁾ The transformed *A. oryzae* niaD300 was then cultivated in DPY medium at 30°C for 90 h with shaking.

Purification of rEndoPG IVs. rEndoPG IVs obtained from the culture filtrate of *A. oryzae* transformants were purified by the following procedures. All operations were carried out below 4°C.

Step 1. Concentration of EndoPG IVs in the culture filtrate.

The culture filtrate (3 L) containing the secreted EndoPG IV was precipitated with ammonium sulfate at 80% saturation and centrifuged at 12,000 × G for 10 min. The resulting pellets were resuspended in 20 mM acetate buffer (pH 5.5), followed by dialysis against the same buffer overnight.

Step 2. DE52 column chromatography. After dialysis, the samples were centrifuged to remove any insoluble materials, and the resulting supernatant was applied to a DE52 column (φ 32 mm × 850 mm) equilibrated with the same buffer. The column was washed with 500 mL of the buffer, and the rEndoPG IV was eluted with a linear gradient of NaCl ranging in concentration from 0 to 62 mM. Fractions containing PG activity were pooled, lyophilized and stored at -20°C.

Step 3. Hydroxyapatite column chromatography. The lyophilized sample was suspended in 2 mM phosphate buffer (pH 6.5) and dialyzed against the same buffer. After dialysis, the samples were centrifuged to remove any insoluble materials, and the resulting supernatant was applied to a hydroxyapatite column (φ 20 mm × 300 mm) equilibrated with the same buffer. The recombinant enzyme was eluted with a stepwise gradient of phosphate buffer (pH 6.5) comprising concentrations of 5, 10, 30, 50 and 100 mM.

PG activity and protein determination. EndoPG activity was determined as described previously. The enzyme reaction for PG was performed at pH 5.0 (50 mM acetate buffer) for the appropriate length of time using a 0.2% polygalacturonic acid (Sigma-Aldrich Corporation, Saint Louis, USA) reaction solution. The activity was assayed by measuring the amount of released reducing sugars using the method of Milner and Avigad.²²⁾ One unit of PG activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugars per min at 30°C (under the assay conditions described). The protein concentration was determined by the Bradford assay²³⁾ using a BSA standard.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli.²⁴⁾ Proteins in the gel were detected by silver staining. A calibration kit (AMRESCO Inc., Cleveland, USA) comprising standard molecular mass markers was employed.

MALDI-TOF MS analysis. Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analyses were performed in the positive ion mode on a VoyagerTM RP HU BioSpectrometryTM Workstation (PerSeptive Biosystems Inc., Framingham, USA). Sinapinic acid (5 mg/mL) or α-cyano-4-hydroxycinnamic acid (5 mg/mL) in 50% CH₃CN/0.1% TFA was used as a matrix. A

Fig. 1. DE52 column chromatography of recombinant EndoPG IV.

rEndoPG IVs were eluted with linear concentration gradient of NaCl from 0 to 62 mM. The fractions collected were 10 mL per test tube. —, Abs (280 nm); ····, enzyme activity; ----, NaCl conc.

ProteoMassTM Peptide and Protein MALDI-MS Calibration Kit (Sigma Chemical Co., Saint Louis, USA) was used for mass calibration, which was performed externally employing apomyoglobin ($[M + H]^+ = 16,952.27$ Da, average mass) and aldolase ($[M + H]^+ = 39,212.28$ Da, average mass) as the proteins. Bradykinin ($[M + H]^+ = 757.399$, average mass) and ATCH ($[M + H]^+ = 2,465.198$, average mass) were used for the sugar chains.

Determination of T_m values. Recombinants were dialyzed against 10 mM acetate buffer (pH 5.0), and then diluted in the same buffer to 0.3 mg protein/mL. CD spectra were measured with a Jasco J-720 spectrophotometer (JASCO Corporation, Tokyo, Japan) at 25°C in a cuvette with a 2-mm path length. The cuvette with the sample was heated to various temperatures (30–70 or 30–90°C) for 5 min, and then the CD spectra were measured. Thermal denaturation curves were obtained by plotting with values at 217 nm in each CD spectrum, and T_m values were determined from each curve.

Preparation of sugar chains from recombinants, and composition analysis. For preparation of sugar chains, rEndoPG IV₁ and IV₂ were treated with endo- β -*N*-acetylglucosaminidase H (EndoH), and then the samples were filtered with UltraFree MC5000NMWL (Millipore KGaA, Darmstadt, Germany). The filtrates (low-molecular-mass fraction) contained the sugar chains from the recombinants.

For analysis of the sugar composition, ion chromatography with a CarboPacTM PA1 column (DIONEX Corporation, Sunnyvale, USA) was employed. The sugar chain samples were hydrolyzed with 2 M TFA (100°C, 3 h), and then analyzed. Fucose, galactose, glucose, mannose and *N*-acetylglucosamine (each 400 mg/mL) were used as standards.

RESULTS AND DISCUSSION

Expression and purification of rEndoPG IVs.

rEndoPG IVs were purified from the culture medium of *A. oryzae* transformed with pNAN8142/EPGIV. The activity in the medium was 6 U/mL after a culture time of 90 h. The rEndoPG IVs in this culture filtrate were precipitated with ammonium sulfate and then purified by DE52 column chromatography on anion-exchange resin, because native

Fig. 2. SDS-PAGE analysis of purified rEndoPG IVs.

Lane M, molecular weight markers; lane 1, rEndoPG IV₂; lane 2, deglycosylated rEndoPG IV₂; lane 3, rEndoPG IV₁; lane 4, deglycosylated rEndoPG IV₁; lane 5, rEndoPG IV₀; lane 6, deglycosylated rEndoPG IV₀.

EndoPG IV from *S. purpureum* has a lower isoelectric point (pI 4.9). The elution pattern obtained with a linear gradient of NaCl indicated four active peaks (Fig. 1). There were two *N*-linked glycosylation motifs in the amino acid sequence of EndoPG IV.⁶⁾ On the other hand, EndoPG I has four *N*-linked glycosylation motifs in the sequence. Four rEndoPG Is (I₁, I₂, I₃, I₄) expressed in *Pichia pastoris* were eluted in order of the decrease in the number of their sugar chains by cation-exchange column chromatography.¹¹⁾ Hence, it was expected that the multiple peaks representing active components would have different numbers of *N*-linked carbohydrate chains. The numbers of sugar chains were predicted and distinguished as rEndoPG IV₂, IV₁ and IV₀ in the order of elution. The last minor active peak in the chromatography elution pattern was assumed to be produced constitutively by PG in *A. oryzae*. The rEndoPG IVs were then purified by DE52 column chromatography and confirmed using SDS-PAGE. Further purification of each active fraction was performed by hydroxyapatite (Gigapite) column chromatog-

Table 1. Purification of recombinant EndoPG IVs.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Culture filtrate*	18824	20916	0.9	100	1.0
Ammonium sulfate Precipitation	12331	717	17.2	65.5	19.1
DE52 PG IV ₂	4716	34.6	136	25.1	151
PG IV ₁	2413	27.7	87.1	12.8	96.8
PG IV ₀	2438	22.7	107	13.0	119
Hydroxyapatite PG IV ₂	2028	5.1	398	10.8	442
PG IV ₁	1724	4.3	401	9.2	445
PG IV ₀	1560	3.8	411	8.3	456

*Culture filtrate 3.0 L.

Fig. 3. MALDI-TOF MS analysis of purified rEndoPG IV₀.

Sinapinic acid was used as a matrix. The mass calibration was performed externally by the use of apomyoglobin and aldolase.

raphy. The three rEndoPG IVs were eluted individually with 50 mM phosphate buffer (pH 6.5). The purified rEndoPG IVs and their deglycosylated enzymes treated with EndoH were then analyzed by SDS-PAGE, and the homogeneities of rEndoPG IV₂, IV₁ and IV₀ were confirmed (Fig. 2). Moreover, the molecular weights of rEndoPG IV₂ and IV₁ were decreased by EndoH treatment, whereas that of rEndoPG IV₀ remained unchanged. In view of the fact that native EndoPG IV had two *N*-glycosylation motifs in the primary structure, it was expected that rEndoPG IV₂ and IV₁ would have two and one sugar chain, respectively, whereas rEndoPG IV₀ had none. The purification steps for rEndoPG IV₂, IV₁, and IV₀ are summarized in Table 1. Their specific activities were ca. 400 U/mg, which approximated that of the native EndoPG IV.

Molecular masses of rEndoPG IVs and structural analysis of the carbohydrate chains

The carbohydrate chains of the three purified rEndoPG IVs were analyzed by MALDI-TOF MS. The molecular mass of rEndoPG IV₀ was 35,098 Da with a molecular ion peak of *m/z* 35,099, which was almost the same as the theoretical value of 35,104 Da based on the primary structure. Furthermore, two molecular ion peaks at *m/z* 35,261 and 35,420 were observed, with a difference of ca.162 (Fig. 3), indicating that one or two hexoses had been added to the protein moiety of rEndoPG IV₀. These results suggested that rEndoPG IV₀ might have an *O*-linked glycosylation lacking in the native type. Woosley *et al.* have reported that recombinants of endoPG C expressed in *A. niger* bear *O*-linked

Fig. 4. MALDI-TOF MS analyses of purified rEndoPG IV₁ and its sugar chains.

Sinapinic acid or α -cyano-4-hydroxycinnamic acid was used as a matrix. The mass calibration was performed externally by the use of apomyoglobin and aldolase for the proteins. Bradykinin and ATCH were used for the sugar chains. (a) MS spectrum of rEndoPG IV₁, (b) MS spectrum of deglycosylated rEndoPG IV₁, (c) MS spectrum of sugar chains cleaved from rEndoPG IV₁ with EndoH.

Fig. 5. MALDI-TOF MS analysis of purified rEndoPG IV₂.

Sinapinic acid was used as a matrix. The mass calibration was performed externally by the use of apomyoglobin and aldolase. (a) MS spectrum of rEndoPG IV₂, (b) MS spectrum of deglycosylated rEndoPG IV₂.

carbohydrate chains.¹⁵⁾ Unlike rEndoPG IV₀, all native forms of EndoPG (EndoPGs I, II, III and IV) had *N*-linked carbohydrate chains. The molecular mass of rEndoPG IV₁ was 36,320 Da after addition of a carbohydrate chain of the M5 type (GlcNAc₂Man₅, 1,216 Da) to the theoretical value (35,104 Da) for EndoPG IV. The multiple peak difference of 162 (Fig. 4(a)) was believed to be due to microheterogeneity of the *O*-linked and *N*-linked carbohydrate chain. The MS spectrum of deglycosylated rEndoPG IV₁ (Fig. 4(b)) after treatment with EndoH showed that the molecular mass was 35,303 Da, suggesting that the deglycosylated rEndoPG IV₁ bore one residue of GlcNAc, and had gained two hexoses from the original number of zero. Furthermore, their sugar chains spliced out with EndoH were recovered, and subjected to MALDI-TOF MS analysis (Fig. 4(c)). This revealed molecular ion peaks of *m/z* 1054 to 1875, with a difference of 162. These *m/z* values correspond to the molecular masses of GlcNAc₁ + hexose₅ and GlcNAc₁ + hexose₁₀, respectively. Therefore, it was confirmed that the recombinants expressed in the host *A. oryzae* had an additional *N*-linked carbohydrate chain bearing five to about ten hexoses. The same analysis was done for rEndoPG IV₂, for which the expected number of carbohydrate chains was two. This revealed a spectrum more complex than that of rEndoPG IV₁ (Fig. 5(a)). The spectrum of deglycosylated rEndoPG IV₂ after EndoH treatment is shown in Fig. 5(b). The molecular ion peak of

Fig. 6. Thermal denaturation curves of purified rEndoPG IVs.

Cuvettes with each sample were heat-treated at each temperature (30–70°C) for 5 min, and then the intensities at 217 nm in the CD spectra were measured. The vertical axis in the graph represents the degree of denaturation, with 100% for complete denaturation. rEndoPG IV expressed in *E. coli* was used for comparison. Symbols: ◆, rEndoPG IV₀; ■, rEndoPG IV₁; ▲, rEndoPG IV₂; ●, rEndoPG IV from *E. coli*.

m/z 35,440 (theoretical value 35,428) indicates that two GlcNAc had been added to the protein moiety of rEndoPG IV₂, while that of *m/z* 35,602 was predicted to be for rEndoPG IV₂ with two GlcNAc + hexose.

The rEndoPG IV₂ had *O*-linked carbohydrate chains with from zero to three hexoses, as was the case for rEndoPG IV₁. The results of splicing out sugar chains from rEndoPG IV₂ were similar to those for rEndoPG IV₁. Finally, sugar composition analysis by ion chromatography showed that the carbohydrate chain recovered from the filter preparation included Gal as well as Man. These results suggested that the *N*-linked carbohydrate chain added to rEndoPG IV is a hybrid type.

The findings described above appear to add important new information about the carbohydrate modification of recombinants when *A. oryzae* is used as a host in a fungus expression system.

Conformational stabilities of the rEndoPG IVs

The thermal denaturation curves of the three rEndoPG IVs were derived on the basis of the intensity of the CD spectrum at 217 nm, indicating a β -strand (Fig. 6). As the rEndoPG IVs expressed in *E. coli* contained incomplete-type disulfide bonds, the thermal denaturation curves showed two steps in the sigmoid curve, suggesting the presence of two rEndoPG IVs with different thermal stabilities.¹¹⁾ The underside of the curve for the recombinant expressed in *E. coli* had a lower *T_m* value due to the incomplete-type disulfide bond (Fig. 6). A similar phenomenon was found for rEndoPG I expressed in *P. pastoris* yeast.¹¹⁾ However, the curves of rEndoPG IV₀ and IV₁ expressed in *A. oryzae* indicated almost the same *T_m* value, 60 and 59°C respectively, with a single step, suggesting that the thermal stability of the rEndoPG IVs was similar to that of the native EndoPG IV. Thus it was thought that the incomplete form of the disulfide bond was absent. The curve for rEndoPG IV₂ showing two steps (*T_m* values 62 and 80°C) was clearly different from those of the other two recombinants. The reason for these two steps was unclear. The thermal stability of rEndoPG IV₂ in terms of activity was similar to those of IV₁ and IV₀. Hence, the

two-step curve was not caused by an incomplete disulfide bond because the T_m value was higher than that of the native type. After deglycosylation with EndoH, the sigmoid curve with two steps changed to a one-step curve, similar to those of rEndoPG IV₀ and IV₁. Therefore, the stability of the secondary structures with a higher T_m value might be improved by the two sugar chains. However, although both components of rEndoPG IV₂ had two sugar chains, the T_m values differed. Thus, the length and position of the *N*-linked carbohydrate chain, which bound to Asn in the protein, may play an important role in the improvement of conformational stability.

Among the rEndoPGs expressed heterologously in *S. purpureum*, we were able to show that the recombinants obtained using an expression system in *A. oryzae* had conformational stability equivalent to that of the native EndoPG IV, with complete set of disulfide bonds, different from the recombinants obtained in *E. coli* and *P. pastoris*.

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