Purification and Characterization of Pectate Lyase I and II from Fungus *Stereum purpureum*

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Two pectate lyases (PLs I and II) were isolated from the culture filtrate of *Stereum purpureum*, the causative fungus of apple silver-leaf disease. The lyases were isolated using DE52 column chromatography, and their molecular masses were estimated to be 48 kDa by SDS-PAGE. After treatment with endo- β -*N*-acetylglucosaminidase (Endo H), their molecular masses decreased equally to 38 kDa, indicating that these PLs are glycoproteins. The *pI* values for PLs I and II were determined to be 4.15 and 4.10, respectively. The amino acid compositions of PLs I and II were very similar, and furthermore the amino acid sequences of the N-terminal 20 residues of the two enzymes were identical. Their optimum pH values, and thermal and pH stabilities were 9.5, up to 50°C, and pH 6–7, respectively. Both PLs I and II required 0.2 mM Ca^{2+} ion for maximum activity. The enzymes were assigned as endo-types because of the detection of oligo-GalUAs as products from the initial reaction. However they mainly formed products less than octa-GalUA in degree of polymerization. *Erwinia carotovora* PL mainly formed products more than octa-GalUA. Therefore they were different in their initial reaction products. As in the case of the *E. carotovora* PL, the final products of *S. purpureum* PL were 4,5-unsaturated di- and tri-GalUA.

During our studies on endopolygalacturonases (endoPG) produced by the fungus *Stereum purpureum*,^{1–3)} which causes silver-leaf symptoms on apple leaves, it was discovered that this fungus produces pectate lyase (PL) in its culture filtrate.⁴⁾ PLs split the glycosidic bond by *trans*-elimination of the hydrogen from the C-4 and C-5 positions of the aglycone portion of the substrate, and are classified fundamentally into two types, endoPL (EC 4.2.2.2) and exoPL (EC 4.2.2.9). Another type of enzyme with terminal mechanism is oligogalacturonate lyase (EC 4.2.2.6) which catalyses the production of 4-deoxy-L-threo-hexos-5-ulose by eliminative removal of the terminal residues reduced from oligo-GalUA. PLs are microbial extracellular enzymes, and those from the genera *Er-*

winia^{5,6)} and *Bacillus*^{7,8)} are well known due to soft-rot symptoms in plants. Furthermore, they are also found in other microorganisms, including *Pseudomonas*,^{9,10)} *Xanthomonas*,¹¹⁾ *Aspergillus*,¹²⁾ *Fusarium*¹³⁾ and *Rhizoctonia*.¹⁴⁾ Although clonings of fungal PLs were reported for *Aspergillus nidulans*¹²⁾ and *F. solani*,¹⁵⁾ their enzymatic properties have been relatively unknown. Recently the role of Ca^{2+} ions in substrate binding using recombinant PL A of *A. niger* was discussed.¹⁶⁾ In PL studies, the elucidation of the enzymatic properties of the fungal type is important. In this paper, we describe the purification of two PLs from fungus *S. purpureum*, and their properties, especially in comparison to those of *Erwinia carotovora*.

MATERIALS AND METHODS

Microorganism and cultivation. The *S. purpureum* Persoon (ASP-4B) used in this study was supplied by the Aomori Apple Experiment Station

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Abbreviations: PL, pectate lyase; DP, degree of polymerization; AIPA, acid-insoluble polygalacturonate; GalUA, galacturonic acid; unsatd. GalUA, 4,5-unsaturated GalUA; Endo H, endo- β -*N*-acetylglucosaminidase.

and maintained by subculture on an agar medium. Medium for PL production was composed of potato decoction liquor (from 150 g of potato/L) containing 10% V8 vegetable juice (Campbell Soup Co.). The mycelia were inoculated into 100 mL of culture in 500 mL Erlenmeyer flask. The cultivation was carried out at 28°C for *ca.* 13 days without shaking.

Preparation of substrates. Pectic acid was prepared from commercial citrus pectin (Wako Pure Chemical Industries, Ltd.) by alkaline saponification. Acid-insoluble polygalacturonic acid (AIPA) was prepared from the commercial citrus pectin by partial acid hydrolysis method of Ozawa.¹⁷⁾ The AIPA was free from neutral sugars and $\overline{DP}$ was estimated to be 43. Unsaturated di-, tri-, tetra-, penta-, hexa-, hepta- and octa-GalUA used as standard for identification of unsaturated oligo-GalUAs produced in HPLC were prepared from AIPA by enzyme degradation with endoPL from *E. carotovora*.^{17,18)}

Enzyme activity assay and protein measurement. The enzyme reaction for PL was done at pH 9.0 for appropriate lengths of times using a 0.1% AIPA reaction solution containing 0.3 mM Ca^{2+} . The activity was assayed by measuring the amount of released 4-deoxy-4,5-dehydro-D-galactosyl groups with absorbance at 235 nm. One unit of PL activity was defined as the amount of the enzyme that produces 1 μ mol 4,5-unsaturated product min^{-1} at 30°C under the assay conditions described. A molar extinction coefficient of $4.7 \times 10^3 M^{-1} cm^{-1}$ was used for calculation.¹⁹⁾ PG and pectinesterase activities were determined according to the previously described methods.¹⁸⁾

The protein concentration was assayed by the method of Lowry *et al.*²⁰⁾ with bovine serum albumin (Sigma) as a standard. Absorbance at 280 nm was used to monitor protein in column chromatography.

Purification of PLs I and II. *S. purpureum* was cultivated for 13 days at 28°C by stationary culture. The culture filtrate obtained was purified by the following procedures. All operations were carried out at temperatures below 5°C.

Step 1. Adsorption on CM52 and DE52 mixture column. The culture filtrate (3.8 L) was di-

luted 3 times with dist. water and adsorbed on a CM52 and DE52 (Whatman, USA) mixture column (6.0 × 22 cm). The adsorbed protein was eluted by 20 mM phosphate buffer (pH 7.5) containing 300 mM NaCl, followed by dialysis against 20 mM phosphate buffer (pH 6.3) and then about 800 mL of the dialysate was lyophilized.

Step 2. 1st DE52 column chromatography.

The lyophilized crude preparation was dissolved with 20 mM sodium phosphate buffer (pH 6.3), and the solution was dialyzed against the same buffer. The enzyme solution was applied to a DE52 column (6.0 × 24 cm) equilibrated with the same buffer. After the column was washed with 2 bed volumes of the buffer, PLs were eluted with the same buffer containing 50 mM NaCl. The active fractions were pooled, concentrated to 50 mL by ultrafiltration, and dialyzed against 20 mM sodium acetate buffer (pH 5.3).

Step 3. Second DE52 column chromatography. The enzyme solution was applied to a DE52 column (3.0 × 80 cm) equilibrated with 20 mM phosphate buffer (pH 6.3). After the column was washed with 1 L of the starting buffer, the adsorbed enzyme was eluted by a linear gradient of 0 to 100 mM NaCl in the same buffer. The two active fractions were individually combined and each was concentrated to 10 mL by ultrafiltration.

Step 4. Gigapite column chromatography.

The enzyme solutions from step 3 were dialyzed against 2 mM sodium phosphate buffer (pH 6.5) and then applied to a Gigapite (Seikagaku Kogyo Co.) column (2 × 30 cm) equilibrated with the same buffer. The column was washed with 250 mL of the starting buffer, PLs were eluted with a linear gradient of 2 to 200 mM of the same buffer. The active fractions were collected and lyophilized.

Step 5. Sephadex G-100 gel filtration. PLs obtained by step 4 were dissolved in 2 mL of 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl and applied to a Sephadex G-100 (Pharmacia LKB) column (3 × 95 cm) equilibrated with the same buffer. After the active fractions were concentrated, they were dialyzed against 20 mM sodium acetate buffer (pH 6.5), lyophilized and stocked at -40°C.

Electrophoretic analysis. Sodium dodesyl sulfate (SDS)-PAGE (12.5% acrylamide gel) was performed by the method of Laemmli.²¹⁾ Proteins in the gel were stained with Coomassie Brilliant Blue R-250 (CBB). A calibration kit (Boehringer Mannheim) was used as standard molecular mass markers.

Isoelectric focusing was done using an Atto AE-3235 apparatus.²²⁾ Slab gels contained 7.5% (w/v) acrylamide, and 0.3% Ampholine (pH 3–10, Pharmacia Biotech.). Isoelectric points was measured using a pI marker protein kit (pH 3–10, Pharmacia Biotech.) as standard pI markers.

Lectin staining of PLs. After SDS-PAGE of PLs, the gel was transferred to a PVDF membrane (Immobilon-P, Millipore, Tokyo) by electroblotting. The membrane was incubated with Peroxidase-ConA, and the protein bands containing ConA-reactive sugar chains were visualized.

Deglycosylation of PLs. Sugar moiety of enzyme was removed by enzymatic hydrolysis with Endo H (New England Biolabs). Twenty μ g of desalted PL I or II were dissolved in 20 μ L of denaturing buffer, boiled for 10 min, and then 3 μ L of 500 mM citrate buffer (pH 5.5) and 150 U/0.3 μ L of Endo H were added. Each reaction mixture was incubated overnight at 35°C, and the deglycosylated PL was lyophilized.

N-terminal amino acid sequencing. The proteins from the SDS-polyacrylamide gel were electrophoretically transferred to a PVDF membrane (Immobilon-P, Millipore). After staining with CBB, the area of PL I or II bands was cut and destained with a 50% solution of EtOH. Each membrane piece was used to determine the amino acid sequence by an automated protein sequencer (PPSQ-10, Shimadzu Co.).

Cultivation of *E. carotovora* and purification of its PL. *E. carotovora* (IFO No. 14082 isolate) used in this study was supplied by the Osaka Fermentation Experiment Station. This was cultivated, with shaking, for 48 h at 27°C. The culture medium used was composed of 0.5% pectin, 0.5% L-glutamine, 0.3% (NH₄)₂SO₄, 0.24% KH₂PO₄, 0.08% Na₂HPO₄, 0.05% MgSO₄·7H₂O and 0.02% CaCl₂.²³⁾ The purification of enzymes did not exceed 5°C. The culture (9 L) was centrifuged

(11,000 g, 15 min, 4°C), concentrated by ultrafiltration (PM-10, Millipore) and dialyzed against 2 mM phosphate buffer (pH 7.0) for 10 h, and the crude preparation was lyophilized. The lyophilizate was dissolved in 20 mM sodium phosphate buffer (pH 7.0) and dialysed against the same buffer. The enzyme solution was applied to a 1st CM52 column (5.3×20 cm) equilibrated with the same buffer. After the column was washed with 2 bed volumes of the buffer, PLs were eluted with the same buffer containing 200 mM NaCl. The active fractions were pooled, concentrated to 20 mL by ultrafiltration, and dialyzed against 20 mM sodium acetate buffer (pH 7.0). The enzyme solution was applied to a CM52 column (3.0×80 cm) equilibrated with 20 mM phosphate buffer (pH 7.0). After the column was washed with 1 L of the starting buffer, the adsorbed enzyme was eluted by a step wise elution of 0 to 200 mM NaCl in the same buffer. The two active fractions (PLs I and II) eluted by 50 mM of NaCl were collected separately and lyophilized. These PLs were homogeneous in SDS-PAGE. In this study, PL I was used.

Analysis of amino acid composition. The amino acid compositions of their enzymes were analyzed by an AccQ TagTM amino acid analysis using an HPLC system (Waters).

Analysis of reaction products. The reaction mixture (1 mL) containing 0.1% AIPA, 0.3 mM Ca²⁺ ion, 50 mM Tris-HCl buffer (pH 9.0) and PL (0.012 U) was incubated at 30°C, and 100 μ L samples were withdrawn at 2, 10 min and 12 h. Each enzyme reaction was stopped by lowering the pH with Dowex 50W×8 and used for HPLC analysis (Waters) using a Shodex Sugar SH1821 column (0.8×30 cm) connected with a Shodex Sugar SH1011P guard column (0.6×3 cm) (Showa Denko Co.). The detections of products were followed at A₂₃₅. The elution was carried out with 0.005N H₂SO₄ at 40°C at 0.5 mL/min.²⁴⁾

RESULTS AND DISCUSSION

Production of PL by S. purpureum.

The production of PL was induced by adding either 0.5% pectin or 10% V8 vegetable juice. As a result, PL activity was significantly higher with

Fig. 1. Time course of PL production by *S. purpureum*.

Potato decoction liquor containing 10% V8 vegetable juice was used as the medium for PL production. Each enzyme activity was assayed under the conditions described in MATERIALS AND METHODS. Symbols: ●, PL activity; △, PE activity; □, PG activity ($\times 10$ U); ▲, pH of the medium.

the addition of the vegetable juice. The PL production as a function of time is shown in Fig. 1. PL activity was initially detected after an 8-day incubation, and attained the highest value after 14 days. Comparatively, PL production preceded PG production; PG showed the maximum activity on the 15th day. Aside from these activities, the filtrate indicated a low activity of pectin lyase.

Purification of PLs I and II.

In the first DE52 column chromatography, two PL isozymes, assigned as PLs I and II, were eluted using 20 mM phosphate buffer (pH 6.3) containing

Fig. 2. First DE52 column chromatography of PLs I and II from *S. purpureum*.

The chromatography was carried out under the conditions described in MATERIALS AND METHODS. Each fraction (15 mL) was assayed for protein ($\circ$, A_{280}) and PL activity ($\bullet$, A_{235}).

50 mM NaCl as shown in Fig. 2. Further elution with 100 mM NaCl yielded additional PL activities, but they were not further purified and characterized in this study. The two PL-active fractions eluted with 50 mM NaCl were pooled and subjected to a second DE52 column chromatography. The resulting PLs I and II fractions were separately pooled. They were purified separately using Gigapite chromatography. PL I or II was weakly adsorbed on the Gigapite column, and was eluted at around 20 mM sodium phosphate buffer (pH 6.5). In the final purification step using Sephadex G-100 gel filtration, each PL-active fraction was eluted as a single peak. The specific activities of

Table 1. Purification of PLs I and II from *S. purpureum*.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Culture filtrate	85000	8700	9.8	1.0	100
DE · CM adsorption	56800	2300	25	2.5	67
1st DE52	43350	250	173	18	51
2nd DE52					
PL I	12700	51	249	25	15
PL II	17100	52	330	35	20
Gigapite					
PL I	10500	35	300	31	13
PL II	13000	42	310	32	14
Sephadex G-100					
PL I	5600	13	448	46	6.6
PL II	8000	19	421	43	9.4

PLs I and II toward AIPA were 448 and 421, respectively. The specific activity of purified *E. carotovora* PL I was 353. A typical purification of enzymes is summarized in Table 1.

Purity, molecular mass, and isoelectric point.

The purity of these enzymes was verified using SDS-PAGE and IEF. The SDS-PAGE analysis of PLs I and II indicated a single band corresponding to a molecular mass of 48 kDa for both (Fig. 3). SDS-PAGE of the purified *E. carotovora* PL I indicated a homogenous enzyme with a molecular mass of 42 kDa (Fig. 3). The analytical IEF showed that PLs I and II also gave a single band, and the *pI* values were estimated as 4.1 for PL I and 4.15 for PL II (Fig. 4). Except for one of the five PLs from *E. chrysanthemi*, which has an acidic *pI*,²⁵⁾ the isoelectric points of all bacterial PL reported to date are alkaline.²⁶⁾

Detection of sugar chain.

The bands of PLs on the SDS-PAGE were subjected to lectin-staining using peroxidase-ConA. After deglycosylation with Endo H, the molecular masses decreased from 48 to 38 kDa (Fig. 5), which indicated that the two PLs are glycosylated proteins that have specific sugar chains for ConA. The PL isolated from *F. moniliformae* was reported also as a glycoprotein with 5% carbohydrate content.²⁷⁾

N-terminal amino acid sequences.

The N-terminal amino acid sequence of the two PLs were identical for 20 amino acid residues as follows: ASVDEVATVGFAGLAGVQGG

This N-terminal amino acid sequence has no similarity at all to the known sequences of pectic enzymes.

Analysis of amino acid composition.

As shown in Table 2, the amino acid compositions of PLs I and II are almost identical. Further, the compositions were similar to that of the purified PL I isolated from *E. carotovora*, and to those of endopolygalacturonase from *S. purpureum* and *Fomitopsis cytisia*.²⁸⁾

Fig. 3. SDS-PAGE of purified PLs I and II from *S. purpureum* and PL I from *E. carotovora*.

Each sample was electrophoresed on an SDS-polyacrylamide gel. Lane M, molecular mass marker proteins; lane 1, *E. carotovora* PL I; lane 2, *S. purpureum* PL I; lane 3, *S. purpureum* PL II. Numbers at the left indicate molecular mass markers in kDa.

Fig. 4. IEF of purified PLs I and II from *S. purpureum*.

Each sample was electrophoresed on a IEF. Lane M, *pI* markers; lane 1, PL I; lane 2, PL II. Numbers at the left indicate *pH* of *pI* markers.

Fig. 5. SDS-PAGE of deglycosylated PLs I and II from *S. purpureum*.

Each sample was electrophoresed on a SDS-polyacrylamide gel. Lane M, molecular mass markers; lane 1, native PL I; lane 2, deglycosylated PL I; lane 3, native PL II; lane 4, deglycosylated PL II. Numbers at the left indicate molecular mass markers in kDa.

Table 2. Amino acid composition of PLs I and II from *S. purpureum*.

Amino acid	<i>S. purpureum</i>		<i>E. carotovora</i>	EndoPG I ^a
	PL I	PL II	PL I	
Mole percent (%)				
Ala	6.8	6.7	10.1	8.0
Arg	2.8	2.7	3.3	1.3
Asp	16.8	16.9	17.2	16.4
Cys	0.6	0.4	3.2	1.5
Glu	6.9	6.9	3.4	3.6
Gly	13.3	13.2	10.6	13.6
His	3.0	3.0	1.6	1.8
Ile	5.7	5.8	6.8	3.9
Leu	8.3	7.8	4.7	6.4
Lys	2.7	2.6	7.5	6.2
Met	0.4	0.4	0.6	0.8
Phe	3.5	3.5	2.6	3.1
Pro	2.3	2.2	3.0	5.7
Ser	6.7	7.0	6.6	7.2
Thr	8.6	9.0	6.8	11.1
Yyr	1.6	1.6	4.3	1.5
Val	9.6	9.7	6.5	6.7

^a From *S. purpureum*.²⁸⁾**Enzyme characteristics.**

The PLs exhibited similar optima pHs of around 9.5. The pH and thermal stabilities were investigated by measuring the residual activities of these enzymes. The PLs were shown to be stable for 10 h at 30°C in the pH range between 6.0 to 7.0, and for 15 min below 50°C at pH 6.0. Although PL I lost its activity, PL II retained 30% of its activity at 70°C.

The effects of Ca²⁺, Mg²⁺, or Mn²⁺ ions and various reagents (EDTA, iodoacetamide, *N*-bromosuccinimide, PCMB, and HgCl₂) on the activities of PLs I and II were also investigated. Both PLs I and II required 0.2 mM Ca²⁺ ion for maximum activities. The addition of Mg²⁺ or Mn²⁺ ion, instead of 0.2 mM of Ca²⁺ ion, decreased the activities of PL I to 9 and 25%, respectively, and for PL II, 13 and 30%, respectively. The activities of PLs I and II were completely inhibited by EDTA as well as many PLs reported so far.⁷⁾ Except for the iodoacetamide, which had no effect, the addition of 1 mM *N*-bromosuccinimide, PCMB, and HgCl₂, completely inhibited the activities of PL. Similarly, *E. carotovora* PL I was seriously inhibited by EDTA, *N*-bromosuccinimide, PCMB,

Fig. 6. HPLC analysis of reaction products by PLs I and II from *S. purpureum* and PL I from *E. carotovora*.

AIPA was used as the substrate for three PLs. The numbers of each peak in (A) indicate the DP of unsatd. GalUA. The reaction times of each diagram in (A), (B) and (C) were 0, 2, 10 min and 12 h from left. (A), *S. purpureum* PL I; (B), *S. purpureum* PL II; (C), *E. carotovora* PL I.

and HgCl₂. However, in contrast, an inhibition of 60% was effected by iodoacetamide, which had no effect on *S. purpureum* PL. *S. purpureum* PLs are different in their inhibitory effects on the activity from *E. carotovora* PL.

Analysis of the reaction products.

Although HPLC analyses of the reaction products, which showed oligo-GalUAs in the initial stage of the reaction (Figs. 6 A, B), indicate that these enzymes function as endo-types, these PLs produced only 4,5-unsatd. oligo-GalUA that were below octa-GalUA. Since this result was different from products in the initial stage of *E. carotovora* PL I, which is a typical endo-type (Fig. 6 C), *S. purpureum* PLs may represent a new class of the enzymes. After 24 h, analysis of the reaction products indicated a major peak for 4,5-unsatd. tri-GalUA (84%) and a minor peak for unsatd. di-GalUA (16%). The ratios of these products were identical for PLs I and II. Thus PLs I and II seem to be incapable of acting on substrates below unsatd. tri-GalUA. In contrast, the final products of *E. carotovora* PL were also unsatd. tri- and di-GalUAs but with a reaction product ratio of 58 and 42%, respectively.

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銀葉病菌のペクチン酸リアーゼ I と II の
精製と性質

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リンゴ銀葉菌の培養口液からペクチン酸リアーゼ I と II (PL I, II) を SDS-PAGE 的に均一成分として単離した。これらの分子質量は 48 kDa で等しく, Endo H 処理によりともに 38 kDa に減少した。また, 等電点はそれぞれ 4.15 と 4.10 であった。N 末端アミノ酸 20 残基は共通であり, アミノ酸組成もきわめて似ていた。これらは至適 pH が 9.5, 熱安定性は 50°C まで, pH 安定性は pH 6.0–7.0 とほぼ同じであり, 0.2 mM Ca^{2+} 存在下で最大活性を示した。反応生成物の HPLC 分析で, 反応初期からオリゴガラクトロン酸を生成したことからエンド型と推定されたが, *E. carotovora* PL による生成物パターンとは異なっていた。また, 反応最終産物は不飽和のジとトリガラクトロン酸であったが, その比率は *E. carotovora* PL の場合とは異なっていた。

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