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PECTINOLYTIC ENZYMES FROM *PSEUDOMONAS MARGINALIS* MAFF 03-01173

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Key Word Index—*Pseudomonas marginalis*; phytopathogenic bacteria; soft-rot-causing pseudomonad; pectin lyase; pectate lyase.

Abstract—Two pectinolytic enzymes were purified from the culture broth of *Pseudomonas marginalis* pv. *marginalis* MAFF 03-01173 with total 33% recovery of the initial activity. From the substrate specificities against pectin and polygalacturonic acid, the requirement of calcium ion for the enzymatic activity, and the N-terminal sequences, the enzymes were identified as pectin lyase and pectate lyase. The M_rs of pectin lyase and pectate lyase were estimated to be 34000 and 43000, respectively, by SDS polyacrylamide gel electrophoresis. Both enzymes showed almost the same pH dependent activity curves with the highest activity at pH 8.3.

INTRODUCTION

Phytopathogenic microorganisms must penetrate carbohydrate barriers of host plant cell walls. Therefore, enzymes which are essential for the degradation of cell wall components are important for their infection to plants. It is well-known that pectinolytic enzymes produced by soft-rot causing bacteria play an important role for the infection to the host plant [1, 2]. There are two types of lyases among pectinolytic enzymes, one is pectate lyase (polygalacturonate lyase, EC 4.2.2.2) which is specific to galacturonic acid residues and the other is pectin lyase (polymethylgalacturonate lyase, EC 4.2.2.10) favouring methyl galacturonate residues. Pectate lyases of *Erwinias* have been extensively studied with respect to pathogenicity, enzymatic properties and gene cloning [3]. Recently, gene cloning and nucleotide sequences of pectate lyases from *Pseudomonas fluorescens* and *Pseudomonas marginalis* have been reported [4, 5], and one of these enzymes has attracted great attention because of the discovery of a new domain motif of a parallel β strands coiled into a large helix with linear stacks of amino acid residues and an asparagine ladder in its molecule [6]. *Erwinias* and *Pseudomonads* produce pectin lyases abundantly under cultivation with inhibitors of DNA synthesis such as nalidixic acid and mitomycin C [7–10]. Nikaidou *et al.* reported, however, that pectin lyases are produced together with pectate lyase by *Pseudomonas marginalis* N6301 cultivated in the medium containing glycerol as a carbon source [11].

To determine the production of lyases, *Pseudomonas marginalis* pv. *marginalis* MAFF 03-01173 was cultivated in the culture medium containing pectin and calcium ion but not antibiotics or glycerol. This paper reports the purification and characterization of two lyases in the medium of *Pseudomonas marginalis* MAFF 03-01173.

RESULTS AND DISCUSSION

Purification

The crude sample was put on a CM-Cellulofine column and chromatographed, resulting in one lyase peak (Fig. 1). By Mono S chromatography, the enzyme activity was separated into two fractions, I and II, as illustrated in Fig. 2(A). These two enzyme fractions were purified by rechromatography with the same conditions [Fig. 2(B) and (C)]. The result of purification is summarized in Table 1. The purification was done simply with four chromatographic steps. The recovery of enzyme activity in each step was

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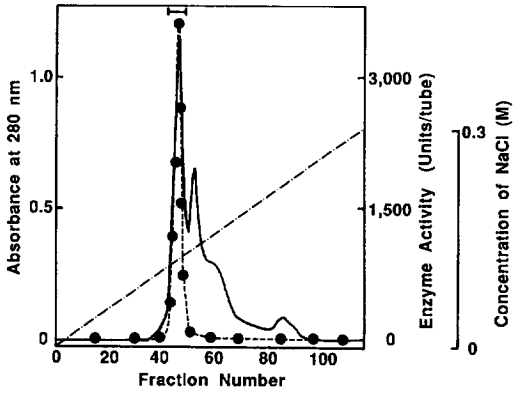

Fig. 1. CM-Cellulofine C-500 ion-exchange chromatography. Pass-through fraction of DEAE-Cellulofine A-500 column was put on a column (1.3 × 30.5 cm) of CM-Cellulofine C-500 equilibrated with 20 mM Tris–HCl buffer, pH 8.0, containing 1 mM $CaCl_2$. Elution was done with a linear gradient of 0 to 0.3 M NaCl in the equilibrium buffer. Fraction volume was 3 ml. —, absorbance at 280 nm; ●, enzyme activity; ---, NaCl concentration.

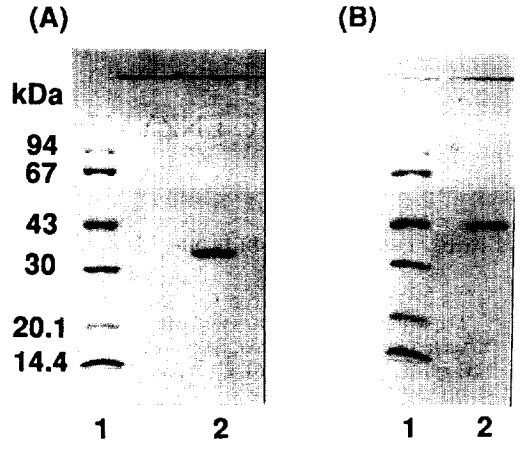

Fig. 3. SDS-polyacrylamide gel electrophoresis of the purified enzymes. (A) Lanes: 1, size marker proteins; 2, enzyme I. (B) Lanes: 1, size marker proteins; 2, enzyme II. Size marker proteins are phosphorylase (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), and α-lactalbumin (M_r 14 400).

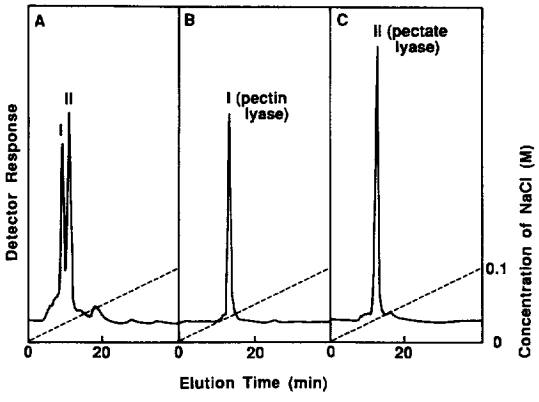

Fig. 2. Mono S ion-exchange chromatography. (A), The active fraction of CM-Cellulofine C-500 ion-exchange chromatography was put on a Mono S HR5/5 column (0.5 × 5 cm) equilibrated with 20 mM Tris–HCl buffer containing 1 mM $CaCl_2$, pH 8.0. Elution was done with a linear gradient of 0 to 0.1 M NaCl in the equilibrating buffer. (B), Enzyme I was rechromatographed under the same conditions as (A). (C), Enzyme II was rechromatographed under the same conditions as (A).

sufficient, indicating that the purification was done properly.

According to Sone *et al.*, in the case of pectin lyase, the production and enzymatic activity was not affected by calcium ion, but the abundant production of pectin lyase was observed only when mitomycin C was added to the medium [10]. However, our results show that enough pectin lyase for the purification was co-produced with pectate lyase in the medium without antibiotics.

Properties

The two purified two enzymes, I and II, indicated a single band on SDS polyacrylamide gel electrophoresis (Fig. 3). The M_rs were estimated to be 34 000 for enzyme I and 43 000 for enzyme II.

The activities of two enzymes were assayed at pH 7–9 using pectin as substrate. Both enzymes showed the highest activity at pH 8.3 and almost the same activity curves.

Table 1. Purification of pectinolytic enzymes produced by *P. marginalis*

Purification step		Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)
Ammonium sulphate precipitate		6700	13 100	1.96	100
DEAE-Cellulofine A-500		398	11 100	28.0	84.7
CM-Cellulofine C-500		11.7	9060	774	69.2
First Mono S	I	2.20	1.460	664	11.1
	II	2.37	4090	1730	31.2
Second Mono S	I	1.69	940	557	7.2
	II	1.62	3400	2100	25.9

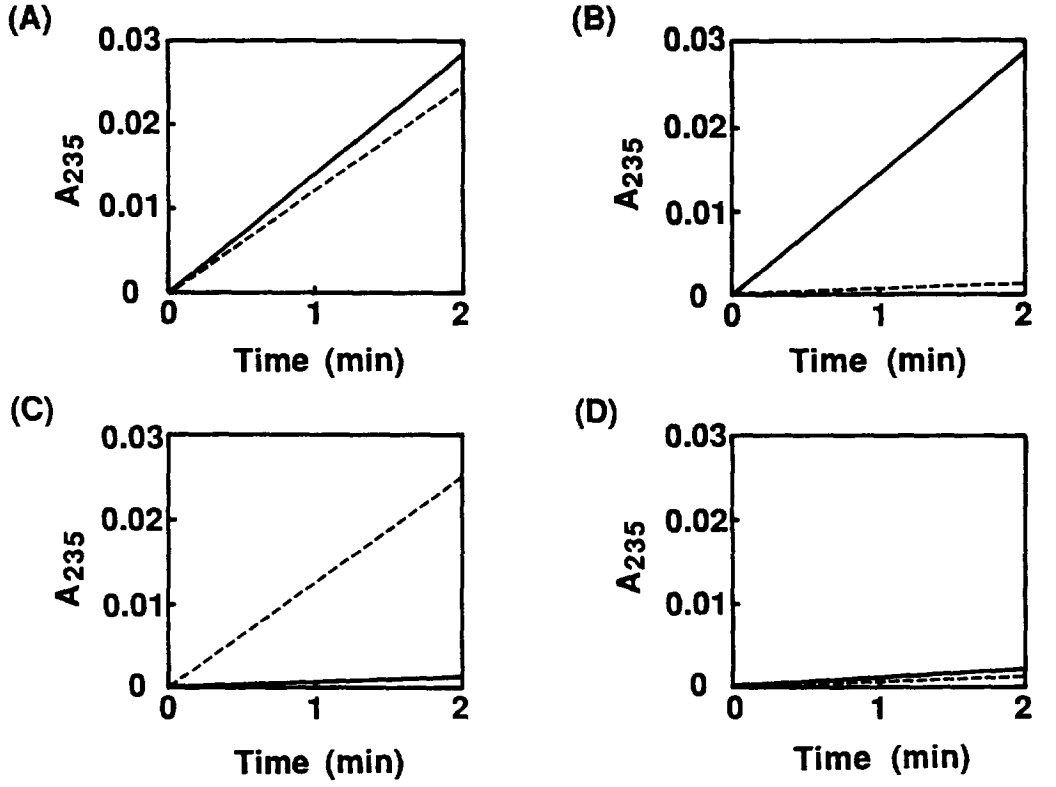

Fig. 4. The lyase activity assay using pectin and polygalacturonic acid. The lyase activity was assayed: (A) with pectin in the presence of 1 mM $CaCl_2$, (B) with pectin in the presence of 5 mM EDTA, (C) with polygalacturonic acid in the presence of 1 mM $CaCl_2$, and (D) with polygalacturonic acid in the presence of 5 mM EDTA. The increase in A_{235} was monitored with a Hitachi 220 spectrophotometer. —, enzyme I; – – –, enzyme II.

For the characterization of purified enzymes, the lyase activity was assayed using pectin and polygalacturonic acid [Fig. 4(A) and (C)] in the presence of $CaCl_2$. Enzyme II increased the absorbance at 235 nm of the reaction mixture in both cases of pectin and polygalacturonic acid used as substrates, indicating the formation of unsaturated bonds at C4-C5 in the galacturonosyl residue at the nonreducing end of polysaccharides. On the other hand, enzyme I only degraded pectin. The result shows that this enzyme can eliminate only glycosidic bonds of methyl galacturonate moiety.

The increase of lyase activity was observed in the culture broth of *P. marginalis*, when $CaCl_2$ was added to the enzyme assay mixture. Therefore, buffer solutions containing $CaCl_2$ were used for all chromatography and enzyme assay. The lyase activity of enzyme II against both pectin and polygalactouronic acid was lost when the reaction mixture was treated with 5 mM EDTA [Fig. 4(B) and (D)]. The requirement of $CaCl_2$ on the lyase activity of enzyme II was similar with that of pectate lyase produced by *P. marginalis* N6301 and *E. carotovora* Er. On the other hand, $CaCl_2$ and EDTA did not affect the lyase activity of enzyme I against pectin. The results obtained from the comparison of the requirement of calcium ion and the substrate specificities clearly indicate that enzyme I is pectin lyase and enzyme II pectate lyase.

N-Terminal sequences

The N-terminal amino acid sequences of enzyme I and II obtained in this study were determined and com-

Enzyme	Origin		Sequence																			
			1									10										20
enzyme I	*P. marginalis*	MAFF 03-01173		S	Y	P	E	S	K	L	T	G	L	I	G	F	A	Q	A	A	K	V	T
pectin lyase[13]	*P. marginalis*	N 6301	M	S	Y	P	E	S	K	L	T	G	L	T	G	F	A	L	A	A	K	V	T
pectin lyase[14]	*E. carotovora*	Er	M	A	Y	P	T	T	N	L	T	G	L	I	G	F	A	K	A	A	K	V	T
			1									10										
enzyme II	*P. marginalis*	MAFF 03-01173	A	D	I	W	L	D	S	A	T	T											
pectate lyase[5]	*P. marginalis*	N 6301	A	D	I	W	L	D	V	A	T	T											
pectate lyase[15]	*E. carotovora*	Er	A	N	T	G	G	Y	-	A	T	T											

Fig. 5. Amino terminal sequences of enzymes I and II.

pared with those from other microorganisms (Fig. 5). The N-terminal amino acid sequence of enzyme I is highly homologous with those of pectin lyases produced by *P. marginalis* N6301 and *E. carotovora* Er. While only four amino acid residues among N-terminal 10 residues are the same between enzyme II and pectate lyase from *E. carotovora*, enzyme II has highly homologous N-terminal sequence with that of pectate lyase from *P. marginalis* N6301. The determination of N-terminal amino acid sequences also indicate that enzyme I is pectin lyase and enzyme II pectate lyase.

Liao reported that Ca^{2+} is essential for the synthesis and secretion of pectate lyase in *Pseudomonads* [4]. Although calcium ion was not added in the culture medium, *P. marginalis* MAFF 03-01173 could produce pectate lyase. This may be due to calcium ion present in tap water (about 0.25 mM) or other factors included in the medium components. Although Ca^{2+} may be essential for the production of pectate lyase in *Pseudomonads* grown in pure culture, other factors may be involved in the production of the enzyme during the invasion of plant tissue. As shown in Table 1, the major lyase activity is due to pectate lyase. Therefore, it is suggested that in soft rotting of vegetables caused by *P. marginalis*, pectate lyase rather than pectin lyase plays a more important role. However, successful penetration of plant tissues by this organism may require the synergistic action of both pectin and pectate lyases.

EXPERIMENTAL

Materials. Citrus pectin and polygalacturonic acid were from Nacalai Tesque (Kyoto, Japan) and Sigma, respectively. All other chemicals were reagent grade.

Bacterial strain and culture medium. P. marginalis MAFF 03-01173 was cultivated in the medium containing 1% Bacto tryptone, 0.5% Bacto yeast extracts, 0.1% citrus pectin, 0.5% NaCl in tap H_2O (pH 7.0) at 30° for 24 hr under shaking (120 rpm).

Enzyme assay. To 0.5 ml of 2% pectin or polygalacturonic acid in 75 mM Tris–HCl (pH 7.5) containing 1 mM $CaCl_2$ 0.5 ml of enzyme soln appropriately diluted with the same buffer was added, and the increase of A at 235 nm was monitored. One unit of the enzyme was defined as the amount that increases 1.0 unit of the A per min at 37°.

Preparation of crude enzyme. After removing bacteria from the culture broth (13.6 l) of *P. marginalis* MAFF 03-01173 by centrifugation, proteins were pptd with 80% sat of $(NH_4)_2SO_4$. The ppts obtained by centrifugation were dialysed against 20 mM Tris–HCl buffer containing 1 mM $CaCl_2$ (pH 8) and insoluble materials after dialysis were removed by centrifugation. The clear but dark brown soln was passed through a DEAE-Cellulofine column to remove a major part of acidic proteins and pigments. The crude sample obtained was used for the further purification.

Chromatography. DEAE- and CM-Cellulofine chromatographies were performed on a medium pressure liquid chromatograph (Yamazen model BPLC-600FC, Kyoto, Japan). A DEAE-Cellulofine A-500 column (2.5 × 33 cm) (Chisso Co., Ltd, Tokyo, Japan) equilibrated with 20 mM Tris–HCl containing 1 mM $CaCl_2$ (pH 8.0) was used to obtain the pass-through fr. A CM-Cellulofine C-500 column (1.3 × 30 cm) (Chisso Co., Ltd, Tokyo, Japan) equilibrated with 20 mM Tris–HCl containing 1 mM $CaCl_2$ (pH 8) was eluted with a 120-min linear conc gradient of NaCl (from 0 to 0.3 M) in the equilibrating buffer. Flow rate was 3 ml min^{-1} and 3 ml frs were collected. Proteins were monitored by A at 280 nm.

Mono S HR 5/5 column chromatography on FPLC (Pharmacia) was carried out by a 40-min linear concn gradient of NaCl (0 to 0.1 M) in 20 mM Tris–HCl buffer containing 1 mM $CaCl_2$ (pH 8.0). Flow rate was 0.5 ml min^{-1}. Protein peaks were monitored by A at 280 nm and collected manually. Rechromatography was performed on the same condition.

Determination of the amount of proteins, amino acid compositions and sequences. The amino acid compositions and the amount of proteins in each step of the purification were determined by amino acid analyses. Hydrolysis of sample was performed with 4 M methanesulphonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako), in a sealed and evacuated tube at 110° for 24 hr. Hydrolysates were analysed by an amino acid analyser (Hitachi model 835, Tokyo, Japan). The N-terminal amino acid sequences of purified enzymes were determined by a protein sequencer (Tosoh model PI 2000, Tokyo, Japan).

Determination of the M_r. The M_r of the purified enzymes was determined by SDS polyacrylamide gel electrophoresis according to the method of ref. [12] using phosphorylase (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), and α-lactalbumin (M_r 14 000) as standard proteins.

REFERENCES

1. Chatterjee, A. K. and Starr, M. P., *Annual Review of Microbiology*, 1980, **34**, 645.
2. Collmer, A. and Keen, N. T., *Annual Review of Phytopathology*, 1986, **24**, 383.
3. Kotoujansky, A., *Annual Review of Phytopathology*, 1982, **25**, 405.
4. Liao, C.-H., *Journal of Bacteriology*, 1991, **173**, 4386.
5. Nikaidou, N., Kamio, Y. and Izaki, K., *Bioscience Biotechnology and Biochemistry*, 1993, **57**, 957.
6. Yoder, M. D., Keen, N. T. and Jurnak, F., *Science*, 1993, **260**, 1503.
7. Tomizawa, H. and Takahashi, H., *Agricultural and Biological Chemistry*, 1971, **35**, 191.
8. Kamimiya, S., Nishiya, T., Izaki, K. and Taka-

hashi, H., *Agicultural and Biological Chemistry*, 1974, **38**, 1072.
9. Itoh, Y., Sugiura, J., Izaki, K. and Takahashi, H., *Agricultural and Biological Chemistry*, 1982, **46**, 199.
10. Sone, T., Sugiura, J., Itoh, Y., Izaki, K. and Takahashi, H., *Agricultural and Biological Chemistry*, 1988, **52**, 3205.
11. Nikaidou, N., Nagamura, T., Kamino, Y. and Izaki, K., *Bioscience, Biotechnology and Biochemistry*, 1995, **59**, 323.
12. King, J. and Laemmli, U. K., *Journal of Molecular Biology*, 1971, **62**, 465.
13. Nikaidou, N., Kamio, Y. and Izaki, K., *Biochemical and Biophysical Research Communications*, 1992, **182**, 14.
14. Ohnishi, H., Nishida, T., Yoshida, A., Kamio, Y. and Izaki, K., *Biochemical and Biophysical Research Communications*, 1991, **176**, 321.
15. Yoshida, A., Matsuo, Y., Kamio, Y. and Izaki, K., *Bioscience, Biotechnology and Biochemistry*, 1992, **56**, 1596.