Facile Isolation of *Endo*-Pectate Lyase from *Erwinia carotovora* Based on Electrostatic Interaction

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Received November 3, 1989; Accepted January 22, 1990

ABSTRACT

Endo-pectate lyase (PATE) from *Erwinia carotovora* was selectively cosedimented with extracellularly produced lipopolysaccharide-lipid complex (LPSLC) through dialysis of the cell free culture broth. The selective isolation of PATE was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The cosedimentation of the PATE with LPSLC was initiated by decreasing conductivity of the solution and terminated at approx 1 m siemens ($mScm^{-1}$). As much as 62% of PATE activity in the culture broth was removed by precipitation. PATE was isolated from the precipitate by gel chromatography. The cosedimentation of PATE with LPSLC was remarkably affected by pH or ionic strength. The addition of polycationic peptide polymyxin B sulfate or a metal chloride affected the interaction. The cosedimentation was diminished by acetylation of the free amino groups of PATE. From these results, it was confirmed that the cosedimentation was induced by electrostatic interaction.

Index Entries: *Erwinia carotovora;* pectate lyase; lipopolysaccharide; enzyme isolation; electrostatic interaction.

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INTRODUCTION

Phytopathogenic species of the genus *Erwinia* synthesize and excrete a variety of pectic enzymes, including *endo*-pectate lyase (PATE, EC 4.2.2.2) when they were grown in the medium using pectin for carbon source (*1,2*) or the medium using other kinds of carbon sources (*3,4*). These tissue macerating enzymes have been investigated biochemically from the industrial and plant pathological points of view (*5,6*). The purification of pectic enzymes has been achieved by the combination of ultrafiltration, ammonium sulfate precipitation, ion-exchange- and gel-chromatographies, and electrophoresis (*7,8*). The purification of PATE based on substrate affinity has been developed (*9*). However, the isolation of pectic enzymes by the materials produced by the same bacterium has not been reported.

We have improved the extracellular PATE production by a two-stage cultivation method (*10*). In a previous paper, we reported that *Erwinia carotovora* produced lipopolysaccharide (LPS) extracellularly (*11*). The association of LPS with enzyme have been studied to elucidate the interactions (*12–14*). However, the interactions between LPS and enzyme are still not clear. Furthermore, the association property of the extracellular LPS and the enzyme excreted by same bacterium has not been reported. In a present paper, we describe the isolation of PATE by cosedimentation with LPS-lipid (L) complex (LPSLC) excreted in the culture broth by *E. carotovora* and characterization of PATE-LPSLC interaction.

MATERIALS AND METHODS

Materials

Citrus pectic acid for PATE assay was purchased from Nacalai tesque, Inc. Kyoto, Japan. The biochemical grade polymyxin B sulfate was from Wako Pure Chemical, Co. Ltd. Osaka, Japan. Sepharose CL-6B for gel chromatography, acrylamide slab gel (gel gradient 8–25%) and the six mol wt standard proteins for electrophoresis were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Bacterium and Cultivation Method

Erwinia carotovora FERM P-7576 was used. The bacterium was cultivated at 28°C by two-stage cultivation method, using pectin and glycerol for carbon sources as reported previously (*10*). *E. carotovora* was initially cultivated in the pectin medium (*1*), and then, after the exponential growth was over (14 h), glycerol and sodium L-glutamate were added up to 1.8% (w/v), respectively, to the medium to continue the cultivation for 14 h.

Dialysis of the Culture Broth

The cells were removed from the culture broth by centrifugation at 9,000×g for 10 min at 4°C, and these centrifugation conditions were employed throughout this study. Cell-free culture broth (40 mL) containing 347 U/mL of PATE activity was dialyzed with cellophane tubing against 2 L of distilled water at 4°C. After dialysis, the resulting precipitate (PPT) was harvested by centrifugation, rinsed with distilled water and lyophilized.

Gel Chromatography

The lyophilized PPT sample (50 mg) containing 165 U/mg of PATE activity was dissolved in 10 mL of 0.05 *M* phosphate buffer (pH 7.0) containing 0.15 *M* sodium chloride. The solution was charged onto a 250 mL Sepharose CL-6B column (2.6 cm×48 cm) previously equilibrated with the same buffer solution at 4°C. Fractions of approx 5 mL were collected every 30 min. The elution profile was obtained by determination of PATE activity, carbohydrate and protein. The fractions containing PATE activity were collected and dialyzed against distilled water with several changes of water, and lyophilized.

Analytical Methods

PATE activity was assayed by a modified method of MacMillan and Vaughn (*15*). The diluted sample solution (0.1 mL, approx 0.5 U/mL) and 10 m*M* $CaCl_2$ solution (0.1 mL) were added to 0.2 *M* ammonium buffer (0.8 mL, pH 9.0). One milliliter of citrus pectic acid (0.5%, w/v) set at pH 7.0 with NaOH was used as a substrate. The enzyme reaction was obtained by incubation at 30°C and stopped after 10 min by addition of 0.2 *M* acetate buffer (2.0 mL, pH 3.8). The enzyme activity was estimated by measuring the optical density at 235 nm. Protein concentration was determined by the method of Bradford (*16*) using crystalline bovine serum albumin as a standard. Carbohydrate concentrations were measured by phenol sulfuric acid method (*17*) using galactose as a reference. 2-Keto-3-deoxyoctonate (KDO) was determined by the thiobarbituric acid method (*18*). Chloroform/methanol (2:1, v/v) extractable material designated as lipid was determined gravimetrically by the same method in the reference (*19*). LPS was estimated by aqueous phenol/chloroform/petroleum ether (PCP) extraction (*20*).

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

The culture broth, PPT free dialysis solution, PPT, F1 (identified as LPSLC) and F2 (PATE). from gel chromatography were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Analysis

was done according to the method of Olsson (*21*). After the electrophoresis, the gel was double stained with the silver stain and the Coomassie Blue stain. The first silver staining step was the modified method for LPS detection (*22*). The second Coomassie Blue staining step for protein was done by the same procedure in the reference (*21*). Photographs were taken after both staining.

Measurement of PATE–LPSLC Interaction

Lyophilized PATE and LPSLC samples fractionated from the PPT by gel chromatography were used. The influence of the addition of reagents on cosedimentation was examined using polycationic antibiotic polymyxin B sulfate or three kinds of metal chloride ($CaCl_2$, $MgCl_2$, and NaCl). PATE solution (50 μL, 1.0 mg/mL) was added to the mixture of LPSLC (75 μL, 1.0 mg/mL) and the varying amounts of polymyxin B sulfate or metal chloride solution, the admixed solution was filled up to 400 mL with water. The reaction mixture was agitated for 1 min at 25 °C, these conditions were sufficient for cosedimentation. The admixed solution was centrifuged to remove the PPT. The supernatant solution was assayed for the PATE activity. The extent of PATE-LPSLC cosedimentation was determined by comparing the PATE activity in the admixed solution and the supernatant solution. The effect of pH on interaction was examined as follows: 50 μg/50μL PATE in 10 or 20 m*M* phosphate buffer was added to 75 μg LPSLC buffer solution (350 μL) in a final vol of 400 μL of the reaction mixture. The extent of cosedimentation was measured by examining PATE activity in the supernatant. The effect of chemical modification on the interaction was investigated by acetylation of PATE. The modification was done by the sodium acetate/acetic anhydride method (*23*). This chemical modification is specific for the free amino groups in the protein. The extent of interaction in the 10 m*M* phosphate buffer was measured by the assay of protein content in the supernatant.

Measurement of LPSLC Aggregation

Various amounts of polymyxin B sulfate, $CaCl_2$, $MgCl_2$ or NaCl was added to LPSLC (75 μg) solutions (final vol 400 μL). The mixture was agitated for 1 min and centrifuged. The extent of LPSLC aggregation was estimated by the assay of carbohydrate content in the supernatant.

RESULTS

Cosedimentation of PATE with LPSLC

The dialysis of the culture broth (10 $mScm^{-1}$ of conductivity) against distilled water induced the sediment formation. The cosedimentation of

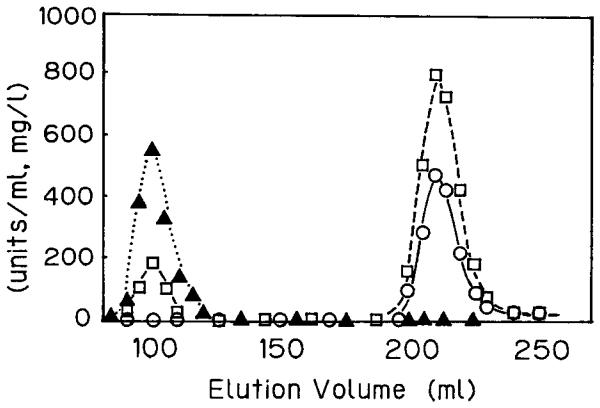

Fig. 1. Elution profile of the dialysis precipitate from gel chromatography. The PPT (50 mg) was charged onto a 250 mL Sepharose CL-6B column. Symbols: ○, PATE activity; ▲, carbohydrate; □, protein.

PATE with LPSLC was stopped at approx 1 $mScm^{-1}$. After 24 h dialysis, 62% of PATE activity in the cell free broth was removed from the solution by the PPT. Cell-free culture broth (40 mL) provided 35.0 mg PPT after rinse with distilled water and lyophilization. The specific PATE activity of the PPT was estimated to be 165 U/mg PPT.

Gel Chromatography

Two peaks were obtained from the PPT by Sepharose CL-6B column chromatography (Fig. 1). The first peak fraction (F1) was eluted just after the void vol. Carbohydrate and a small amount of protein were found in this peak fraction. After extensive dialysis, the solution was lyophilized and 23.2 mg of LPSLC was obtained from 50.0 mg PPT. The second peak fraction (F2) was identified as PATE, since it contained both protein and PATE activity. After dialysis and lyophilization, F2 fraction contained 8.3 mg PATE, of which specific activity was 710 U/mg protein.

SDS-PAGE

The silver strained SDS-PAGE gel is shown in Fig. 2A. The silver strain method clearly revealed the LPS band in a culture broth (lane 1), PPT free dialysis solution (lane 2), PPT (lane 3), and F1 fraction from gel chromatography (lane 4). The double stained gel (Fig. 2B) indicates the protein composition of the samples. When the PPT was electrophoresed by the same methods and stained by Coomassie Blue, there were no distinct protein bands except PATE (data not shown). Thus, the PPT was composed of single protein and LPS (lane 3). The purity of the isolated PATE was confirmed by the major protein band revealed in F2 fraction (lane 5) with mol wt 42,000.

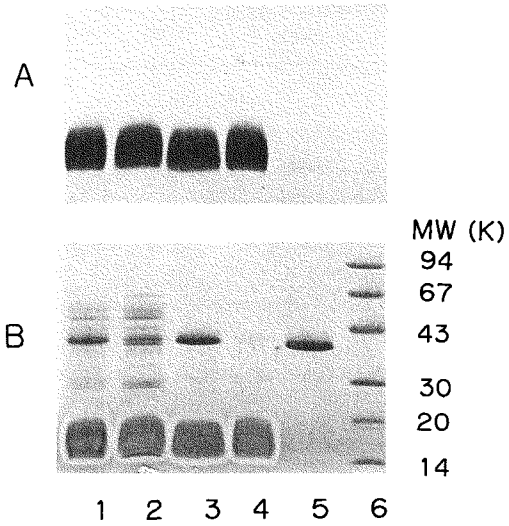

Fig. 2. SDS-PAGE (A) The silver stained gel. (B) The double stained gel; the gel was stained with the silver and the Coomassie Blue staining. Lanes 1, Cell-free culture broth; 2, Dialysis precipitate (PPT) free solution; 3, PPT; 4, LPSLC (F1) fraction obtained from the PPT by gel chromatography; 5, PATE fraction (F2); 6, Low mol wt protein standard (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin).

Table 1
Composition of PPT and LPSLC

	PPT	LPSLC
Component	(% dry wt)	
Protein	25.0	4.2
Lipid[a]	34.2	45.2
Lipopolysaccharide	32.2	41.2
Carbohydrate	10.6	15.1
KDO[b]	2.5	3.4

[a]The materials extractable by $CHCl_3$:CH_3OH=2:1.
[b]2-keto-3-deoxyoctonate.

Composition of the PPT and LPSLC

The components of the PPT and LPSLC were shown in Table 1. The percentages of LPS in PPT and LPSLC estimated by PCP extraction were 32 and 41%, respectively. KDO, which is a specific component of LPS, in both fractions, were 2.5 and 3.4%, respectively.

Cosedimentation of PATE with LPSLC

The pH of the sample solution and the addition of metal chloride to PATE-LPSLC admixed solution showed little effect on PATE activity under

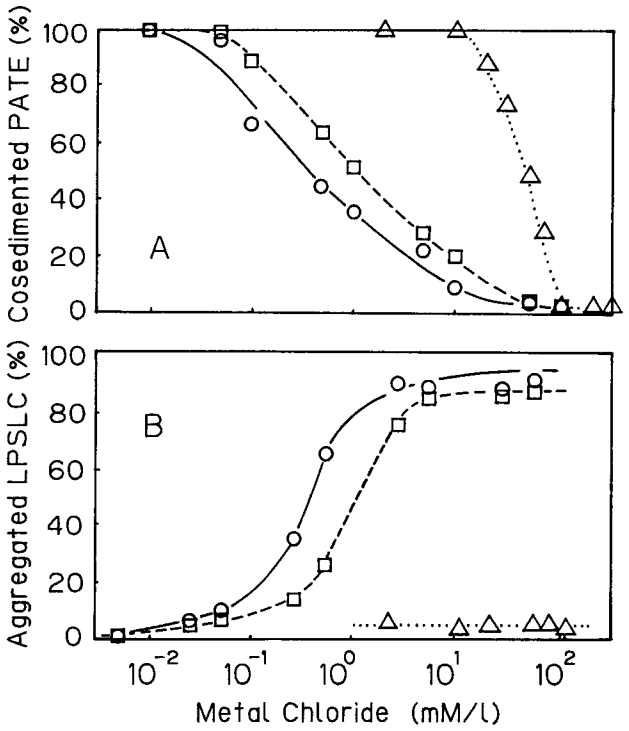

Fig. 3. Influence of metal chloride. (A) on PATE-LPSLC cosedimentation (B) on LPSLC aggregation. Symbols: ○, $CaCl_2$; □, $MgCl_2$; △, NaCl.

the assay method used in this study. As shown in Fig. 3A, the cosedimentation of PATE with LPSLC was remarkably affected by the addition of metal chloride. The similar results were obtained with addition of polymyxin B sulfate. The 0.35 weight ratio of polymyxin B sulfate to LPSLC disrupted the cosedimentation completely. The cosedimentation was also sensitive to the monovalent salt such as NaCl. The cosedimentation was disrupted completely in the presence of 100 m*M* metal chloride of both monovalent and divalent metals. The interaction of PATE with LPSLC was sensitive to the divalent metal chloride at the approx 1000-fold range of the concentration. On the other hand, the effect of NaCl was observed at the 10-fold range of the concentration. The effect of divalent metal chloride on PATE-LPSLC cosedimentation was different from that of NaCl.

As illustrated in Fig. 4, pH of the solution affected the cosedimentation. In 10 m*M* buffer solution, the transition occurred at around pH 10.5, which is close to the isoelectric point of PATE (*7*). In addition, the pH at which the transition of cosedimentation occurred was also affected by the concentration of the buffer. When the free amino groups of PATE were modified by acetylation, the interaction of PATE with LPSLC in 10 m*M* phosphate buffer was significantly reduced.

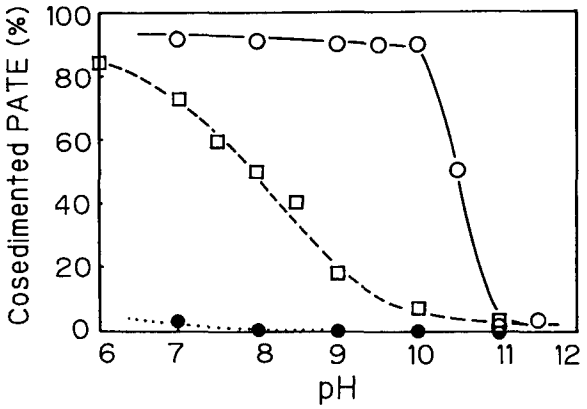

Fig. 4. Effect of pH on PATE-LPSLC cosedimentation. Symbols: ○, 10 m*M* phosphate buffer; □, 20 m*M* buffer; ●, acetylated PATE in 10 m*M* buffer.

Aggregation of LPSLC

The effect of reagents on aggregation of LPSLC were illustrated in Fig. 3B. LPSLC was aggregated in the presence of $CaCl_2$ or $MgCl_2$. In addition, polymyxin B sulfate presented the same results. The curves were quite symmetrical with PATE cosedimentation curve (Fig. 3A). On the other hand, NaCl showed little effect on LPSLC aggregation.

DISCUSSION

The isolation of enzyme as LPS-containing material from cells or culture broth has been reported with *Salmonella typhimurim* (*12*) and *Pseudomonas aeruginosa* (*24,25*). The purification of the enzyme from enzyme-LPS complexes were achieved in these studies. The investigation of the enzyme-LPS interaction is important for the isolation of the enzyme from the complexes. The association property of lysozyme with LPS has been studied to elucidate the relation of the enzyme action on the cell surface (*13*) or to characterize the interaction (*14*). However, the interaction of enzyme with LPS has not been extensively studied.

The interaction of the protein with LPS has been studied using the membrane protein and LPS obtained from the cells by extraction. The membrane protein and LPS were tightly associated and not fractionated by gel chromatography (*26*). The protein-LPS complexes obtained from the culture broth were also tightly associated. The tight association is supposed to be induced by hydrophobic interactions (*24,27*). on the other hand, the results in this paper showed the evidence that the interaction of PATE with LPSLC was not tight. PATE was isolated from PPT by Sepharose-gel chromatography. As shown in Fig. 3, the experiments on interaction revealed that the cosedimentation was affected not only by multivalent

materials such as $CaCl_2$ or $MgCl_2$, but also by monovalent metal chloride NaCl. The cosedimentation curves were symmetrical with the LPSLC aggregation curves except NaCl. NaCl showed effects on cosedimentation, however, little effects in LPSLC aggregation at any concentrations. The disruption of PATE-LPSLC interaction was not limited to ionic strength of the solution. The effect of divalent metal chloride on PATE-LPSLC cosedimentation may be different from that of NaCl.

It has been reported that the reconstitution of membrane protein and LPS complex of *Salmonella minnesota* was obtained in a buffered saline; in addition, the presence of $MgCl_2$ enhanced the LPS-protein interaction (*28*). Presumably, when the hydrophobic interaction is dominant, multivalent cations are effective for the association. These results are distinct from the cosedimentation property reported in this study. The interaction of PATE and LPSLC may be different from that of membrane protein and the LPS. The role of divalent metal chloride on aggregation of LPS has been discussed by the ion-exchange reaction (*29,30*). KDO and phosphate moiety, in the salt form should completely be replaced by the multivalent cations, then, negative charge of LPS is reduced. From this point of view, the ion-exchange reaction of LPS in LPSLC affected the cosedimentation with PATE aggregation of LPSLC. Thus, the aggregation of LPSLC may be induced by the ion-exchange reaction of the multivalent cations. The cause of the wide range effects on cosedimentation by divalent metal chloride is presently unknown, the more likely hypothesis is that the aggregation of LPSLC reduced the ion-exchange reaction.

The electrostatic interaction between PATE and LPSLC was supported by the effect of pH or ionic strength on cosedimentation (Fig. 4). In addition to these results, interaction was diminished by acetylation of the amino groups in PATE. Therefore, it was confirmed that the electrostatic interaction between the negative charge of LPS and the positive charges of PATE is essential for the cosedimentation of PATE with LPSLC.

The facile isolation method for PATE from the culture broth provides the potential application of PATE from *E. carotovora*.

ACKNOWLEDGMENT

The authors wish to thank Eriberta N. Navera for reviewing the manuscript.

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