Purification and Characterization of *Endo*-Pectate Lyase from *Bacillus macerans*

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Bacillus macerans **produced an extracellular *endo*-pectate lyase when cultivated with grated potato tuber. The enzyme activity was markedly activated by 1 mM calcium or manganese ion, but completely inactivated by ethylenediaminetetraacetate (EDTA). The activity was maximum at pH 9.0 and 60°C. The enzyme activity was stable up to 50°C and 55°C for 10 min at pH 9.0 in the absence and presence of calcium ion, respectively, and between pH 6.5 and 9.5 for 2 hr at 37°C. The enzyme had a molecular weight of 35,000 and a p*I* of 10.3. The Michaelis constant and maximum reaction velocity of the enzyme for polypectate were 0.11% and 74 μmol of unsaturated galacturonate formed per min per mg protein, respectively. The enzyme also attacked pectin that contained 4.3% methoxyl groups. The enzyme was stable in 100 mM ammonium chloride buffer (pH 9.0) for a month at 10°C, but 70% of its activity was lost in 20 mM ammonium chloride buffer (pH 9.0) after 24 hr at 10°C.**

Bacillus macerans IFO 3490 is one of the best-known bacteria for macerating plant tissue. In previous papers,[1,2] we reported that *B. macerans* produced pectate lyase (EC 4.2.2.2) extracellularly, probably related to its plant maceration activity.

Recently the application of pectate lyase has advanced in various fields such as pretreatment of pectic wastewater[23] and biochemical pulping.[14]

There were a number of studies on pectate lyases from soft-rot *Erwinia* that cause plant diseases.[3-16] However, few papers were on *Bacillus* pectate lyases.[17-23] The pectate lyase of *B. macerans* had not been purified and characterized.

This paper reports the purification and properties of this enzyme.

Materials and Methods

Microorganism and cultivation. Bacillus macerans IFO 3490 was precultured at 34°C for 2 days in 2% Kyokuto Seiyaku Nutrient Broth. A medium for enzyme production, consisting of 5% grated potato, 0.3% ammonium sulfate, 0.05% yeast extract, and 0.05% magnesium sulfate, was sterilized at 120°C for 20 min. Potato was grated, washed exhaustively with water to eliminate starch granules and dehydrated by a cloth bag. Trisaminomethane (Tris) solution was sterilized separately and added to the medium to give a final concentration of 0.5%. Ten ml of precultured fluid was added to 1-l of the enzyme production medium in a 5-l conical flask and the mixture was incubated at 30°C for 2 days on a shaker. The initial pH of the medium was about 8.5.

Chemicals. All chemicals used in this work were commercial products. Sodium polypectate, serum albumin, and molecular weight markers were obtained from Sigma Chemical Co., St. Louis, U.S.A. DEAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. CM-Cellulofine was from Seikagaku Kōgyo K.K., Tokyo, Japan. Ampholine was from LKB, Stockholm, Sweden. Pectin containing 4.3% methoxyl groups, or 26.3% esterified galacturonate residues was purchased from Katayama Kagaku K.K., Osaka, Japan.

Enzyme assay. Sodium polypectate solution (0.83%) containing 20 mM ammonium chloride was warmed at 60°C and stirred for 10 min, cooled at 37°C, adjusted to pH 9.0, and centrifuged at 13,000 × *g* for 20 min to obtain a clear homogeneous substrate solution. Enzyme solution was diluted with 0.1 M ammonium chloride buffer (pH 9.0) to an activity of about 0.5 units per ml. A reaction mixture composed of 0.2 ml of enzyme solution, 0.1 ml of 0.5 M ammonium chloride buffer (pH 9.0), 0.1 ml of 0.01 M calcium chloride, and 0.6 ml of the above polypectate solution was incubated at 37°C for 1 min, and the reaction was stopped by adding either 4 ml of 0.2 M sodium acetate buffer (pH 3.8) or 4 ml of 1 mM sodium ethylenediaminete-

traacetate (EDTA). The amount of unsaturated galacturonate released was measured from the increase in absorbance at 235 nm. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of unsaturated galacturonate per min under the above conditions. The molecular extinction coefficient of unsaturated product at 235 nm is 4600.[11]

Protein measurement. Protein was measured by the method of Hartree,[30] with bovine serum albumin as the standard. In chromatographic steps and in isoelectric focusing, protein was followed by its absorbance at 280 nm. The value $E_{1\,cm}^{1\%}$ at 280 nm for the purified pectate lyase was 10.3.

Measurement of isoelectric point. Isoelectric focusing was done by the method of Vesterberg[31] at 15°C for 72 hr in a LKB column of 110-ml capacity with a sucrose density gradient containing a mixture of carrier Ampholines [Ampholine pH 4–6 (40%): Ampholine pH 3.5–10.0 (40%): Ampholine pH 9–11 (20%) = 1 : 2 : 3 (by volume)]. After focusing, effluent from the column was fractionated in test tubes. Each fraction (2 ml) was measured for pH at 20°C.

Electrophoresis and measurement of molecular weight. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done in 11.2% gel containing 0.1% SDS by the method of Weber and Osborn described by Hayashi *et al.*[32] The molecular weight of the denaturated enzyme was estimated by comparing its migration rate with those of the standard protein markers. The molecular weight of the native enzyme was estimated by the method of Hedrick and Smith.[36] The gels were stained with Coomassie Brilliant Blue G-250 by the method of Diezel *et al.*[33]

Comparison between the rate increase in fluidity and the rate of increase in unsaturated products. Viscosimetric and spectrophotometric assays were done in parallel at 37°C. Viscosity was measured using a 10-fold scale of volume of the reaction mixture as described in the standard assay. Spectrophotometric assay was done in the same manner as described in the standard assay. Comparison ratio (CR) was computed by Tam's method.[34] The CR was expressed by dividing the rate of increase in fluidity by the rate of increase in unsaturated product concentration:

$$CR = \frac{\Delta \text{ fluidity } (1/\eta_{sp})/\text{min}}{\Delta\ \mu\text{mol unsaturated product/ml} \cdot \text{min}}$$

ml: volume of reaction mixture

Procedure for purification. The culture filtrate (850 ml) was brought to 80% saturation by adding solid ammonium sulfate. The resulting precipitate was kept at 5°C for 1 hr, collected by centrifugation, dissolved in 150 ml of 20 mM ammonium chloride buffer (pH 9.0), and dialyzed three times against the same buffer (100-fold volume). The dialyzate (227 ml) was put on a DEAE-Sephadex A-50 column (1.3 × 10 cm) equilibrated with the same buffer. After washing with the same buffer, the passed and washed fractions (239 ml) were collected and put on a CM-Cellulofine column (1.3 × 10 cm) previously equilibrated with the same buffer. After it was washed with the same buffer, the enzyme was eluted with a linear gradient of 200 ml of ammonium chloride buffer (pH 9.0) from 0.02 M to 0.3 M. The active fractions (93 ml) eluted around 0.05 M ammonium chloride, were pooled and dialyzed against 0.02 M ammonium chloride buffer. The dialyzate (92 ml) was again put on a column (1.3 × 10 cm) of CM-Cellulofine previously equilibrated with the same buffer. The column was washed with the same buffer and the enzyme was eluted in the same manner as in the previous step. The active fractions (96 ml) were pooled and dialyzed against 0.1 M ammonium chloride buffer (pH 9.0).

Results and Discussion

Enzyme production

In the cultivation of *B. macerans*, grated potato tuber, one of the medium components, stimulated the production of pectate lyase more strongly than pectin. This bacillus required strong aeration for the production of pectate lyase, and therefore a conical flask loosely closed by cotton stopper was used and shaken on a shaker at 200 rpm.

Enzyme purification

The elution profile from the first CM-

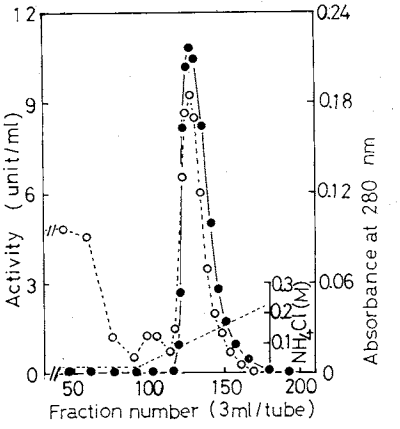

Fig. 1. Elution Profile of *B. macerans Endo*-Pectate Lyase on the First CM-Cellulofine Column.

The experimental details are described in the text. —●—, activity; ---○---, $A_{280\,nm}$; ------, NH_4Cl.

Table I. PURIFICATION OF *Endo*-PECTATE LYASE FROM *Bacillus macerans*

Step	Total activity (U)	Specific activity (U/mg)	Yield (%)
Culture filtrate	1,512	1.6	100
Ammonium sulfate	965	13.3	64
DEAE-Sephadex A-50	837	30.4	55
CM-Cellulofine (1)	714	55.0	47
CM-Cellulofine (2)	423	59.2	28

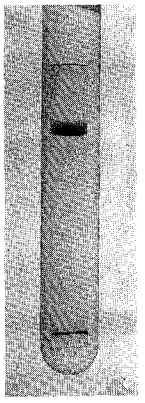

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of Purified *B. macerans Endo*-Pectate Lyase.

The experimental details are described in the text. Enzyme protein used was about 40 μg.

Cellulofine is shown in Fig. 1. A typical purification of the enzyme is summarized in Table I. The enzyme was purified approximately 40-fold from the culture filtrate with an overall yield of 28%.

Homogeneity and molecular weight

As shown in Fig. 2, the purified enzyme gave a single band of protein on SDS-polyacrylamide gel electrophoresis. The molecular weight of the denatured enzyme was measured by SDS-polyacrylamide gel electrophoresis, by which it was defined that the enzyme has a molecular weight of 35,000 (Fig. 3). The molecular weight of the native enzyme is smaller than 45,000. Therefore, the enzyme has no subunits.

The molecular weight of the enzyme is

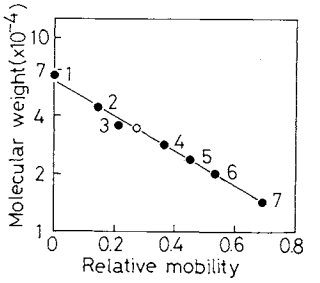

Fig. 3. Molecular Weight Estimation of *B. macerans Endo*-Pectate Lyase by SDS-Polyacrylamide Gel Electrophoresis.

The markers used are: 1, bovine albumin (molecular weight 66,000); 2, egg albumin (45,000); 3, glyceraldehyde-3-phosphate dehydrogenase (36,000); 4, carbonic anhydrase (29,000); 5, Trypsinogen (24,000); 6, soybean trypsin inhibitor (20,100); 7, α-lactalbumin (14,000). Open circle corresponds to *endo*-pectate lyase.

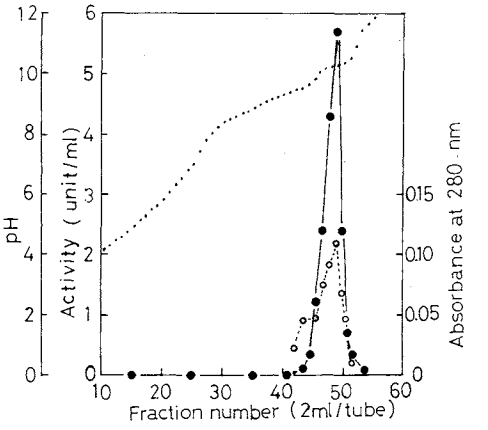

Fig. 4. Isoelectric Focusing of *B. macerans Endo*-Pectate Lyase Achieved by The First CM-Cellulofine Chromatography.

The experimental details are described in the text. —●—, activity; ---○---, $A_{280\,nm}$; ------, pH.

intermediate among those of bacterial *endo*-pectate lyases, as the molecular weight of *Erwinia aroideae* enzymes ranged from 36,000 to 38,000,[7] *Erwinia carotovora* p*I*-isozymes from 28,000 to 34,000,[11,13] 41,000 of *Streptomyces nitrosporeus* enzyme,[25] and 33,000 of *Bacillus* sp. enzyme.[23]

Isoelectric point

As shown in Fig. 4, the enzyme activity was found as a single peak and the isoelectric point

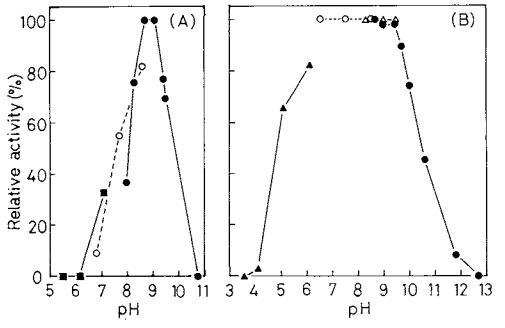

Fig. 5. Effects of pH on the Activity (A) and Stability (B) of *B. macerans Endo*-Pectate Lyase.

The reaction mixtures in the (B) containing 18 μg per ml of the enzyme protein and 0.05 M each buffer were left at 37°C for 2 hr and their remaining activities were assayed at pH 9.0. Buffers used are: —●—, glycine–NaCl–NaOH; —△—, NH_4Cl–NaOH; ---○---, Tris–NaCl–HCl; —■—, sodium phosphate; —▲—, acetic acid–NaOH.

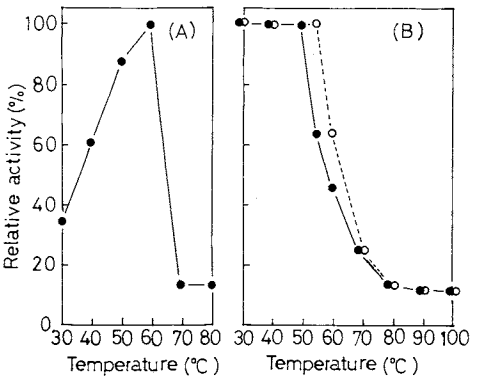

Fig. 6. Effects of Temperature on the Activity (A) and Stability (B) of *B. macerans Endo*-Pectate Lyase.

The reaction mixtures in the (B) containing 4.2 μg per ml of the enzyme protein and 0.23 M ammonium chloride buffer (pH 9.0), or 3.2 μg per ml of the enzyme protein, 0.14 M the same buffer, and 2.5 mM calcium ion were left at each temperature and pH 9.0 for 10 min, and their remaining activities were assayed under the standard conditions. Signals used in the (B) are: ---○---, with 1 mM calcium chloride; —●—, without 1 mM calcium chloride for a time of heating.

was 10.3.

Some enzyme properties

Enzyme stability. The enzyme was stable at 10°C for a week in the culture filtrate and for a month in the 0.1 M ammonium chloride buffer (pH 9.0). The enzyme kept in the 0.1 M ammonium chloride buffer (pH 9.0) lost 87% and 100% of its initial activity after dialysis at 10°C against deionized water for 8 and 24 hr, respectively. When dialyzed against 0.02 M ammonium chloride buffer (pH 9.0), the enzyme lost 40% of its activity after 10 hr and 70% after 24 hr.

Effects of pH on activity and stability. As shown in Fig. 5, the enzyme was most active around pH 9.0 at 37°C and stable at 37°C for 2 hr between pH 6.5 and 9.5. The optimal pH of the enzyme was similar to those of other bacterial pectate lyases such as pH 8.3 to 9.6 of *Bacillus polymyxa* enzyme,[18] 9.0 of *Bacillus* sp. enzyme,[23] 9.0 to 9.2 of *Streptomyces fradiae* enzyme,[24] and 9.0 of *Erwinia atroseptica* enzyme.[10]

Effects of temperature on activity and stability. As shown in Fig. 6, the enzyme had a maximal activity at 60°C and was stable up to 50°C or 55°C at pH 9.0 for 10 min in the absence or presence of 1 mM of calcium ion, respectively. The enzyme retained 11% of its initial activity after heating at 100°C for 10 min. This is supported by the following facts. When the reaction mixture containing polypectate, 1 mM calcium ion and the enzyme was heated at 100°C for 10 min, its viscosity decreased. But the mixture which contained the same components, with the addition of 1 mM EDTA instead of 1 mM calcium ion, had no change in its viscosity. The optimal temperature of pectate lyase from *B. macerans* was similar to those of other bacterial enzymes such as *Bacillus pumilus* enzyme,[19] *Bacillus* sp. enzyme,[23] and one of *Erwinia carotovora* pI-isozymes.[12]

Effects of substrate concentrations. The Michaelis constant (*Km*) and maximum reaction velocity (V_{max}) for polypectate were calculated from the Lineweaver–Burk plots in Fig. 7. The *Km* and V_{max} were 0.11% and 74 μmol of unsaturated galacturonate formed per min per mg protein, respectively.

Effects of various compounds. The effects of various compounds on the enzyme activity are summarized in Table II. Monovalent cations such as potassium and sodium ions did not

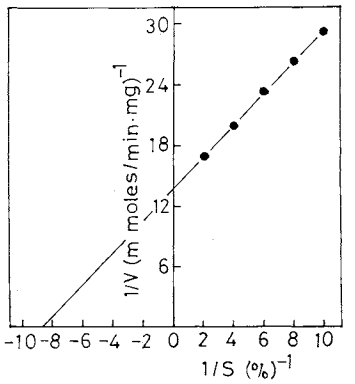

Fig. 7. Lineweaver–Burk Plots of *B. macerans Endo*-Pectate Lyase for Polypectate.

Table II. Effects of Compounds on Pectate Lyase Activity

Compounds	Activity (%)
None (H_2O)	100
NaCl	102
KCl	97
$CaCl_2$	498
$MnSO_4$	612
$MgSO_4$	98
$BaCl_2$	52
$Zn(CH_3COO)_2$	11
CH_2ICOOH	103
EDTA	0

Reaction mixtures containing 1 mM of each compound instead of calcium chloride were used under the standard conditions.

affect the activity, while divalent cations showed positive or negative effects. Calcium and manganese ions enhanced the activity 5- and 6-fold, respectively. Magnesium ion had no effect but barium and zinc ions inhibited the activity by 50% and by 90%, respectively. The enzyme activity was maximum at 1.0 mM calcium ion. The activity was completely inhibited by 1 mM EDTA but not at all by 1 mM iodoacetic acid.

Effects of divalent cations on pectate lyase activities differed among their sources,[17,18,21,25,27-29] species[7,11,12] and individual isozymes.[12,18]

Mode of action. Tam[34] reported that CR value was within 0.15 for *exo*-type enzymes but between 2.04 and 2.99 for *endo*-type enzymes.

Table III. Activities of *Bacillus macerans* *Endo*-Pectate Lyase for Polypectate and Pectin

Substrate	Relative activity (%)		
	Calcium ion		EDTA[d]
	Presence[c]	Absence	
Polypectate	100[a]	20	0
Pectin[b]	98	98	0

[a] Enzyme activity assayed under the standard condition was chosen as 100%.
[b] Pectin, containing 4.3% methoxyl groups, was used as substrate.
[c] Final calcium ion concentration was 1 mM.
[d] EDTA was added instead of calcium ion (final concentration 1 mM).

On the basis of their CR values ranging between 3.60 and 3.95, Tanabe *et al.*[11] classified four pectate lyase p*I*-isozymes from *Erwinia carotovora* as enzymes with *endo*-type action. The calculated CR value of 3.3 for the pectate lyase from *B. macerans* indicated that this enzyme is classified as an *endo*-type enzyme.

Action on pectin. Pectin containing 4.3% methoxyl groups, was attacked by the *endo*-pectate lyase of *B. macerans*. The results are summarized in Table III, in which the data for pectin are compared with those for polypectate. The methoxyl group in the pectin was assayed by Wood's method.[35] The pectin has 73.7% of the same molecular structure that appeared in the polypectate, and the *endo*-pectate lyase attacked the polypectate in a random manner. Thus, the pectin is degraded by this enzyme.

Almost all *endo*-pectate lyases reported required divalent cations for their function. There is a report[18] in which the authors suggest that calcium-bridged oligogalacturonides may be degraded by *endo*-pectate lyase. Calcium ion commonly activated the action of *endo*-pectate lyases for polypectate.

References

1) Y. Miyazaki and K. Nakao, *Bull. Fac. Agr. Saga*

Univ., **47**, 11 (1979).
2) Y. Miyazaki, Y. Yamashita and T. Inaba, Abstracts of Papers, Annual Meeting of the Agricultural Chemical Society of Japan, Fukuoka, April, 1980, p. 42.
3) A. Kotoujansky, *Ann. Rev. Phytopathol.*, **25**, 405 (1987).
4) A. Collmer and N. T. Keen, *Ann. Rev. Phytopathol.*, **24**, 383 (1986).
5) K. Okamoto, C. Hatanaka and J. Ozawa, *Agric. Biol. Chem.*, **28**, 331 (1964).
6) A. Garibaldi and D. F. Bateman, *Physiol. Plant Pathol.*, **1**, 25 (1971).
7) S. Kamimiya, Y. Itoh, K. Izaki and H. Takahashi, *Agric. Biol. Chem.*, **41**, 975 (1977).
8) S. Tsuyumu, *Nature*, **269**, 237 (1977).
9) S. Tsuyumu, *J. Bacteriol.*, **137**, 1035 (1979).
10) P. Quantick, F. Cervone and R. K. S. Wood, *Physiol. Plant Pathol.*, **22**, 77 (1983).
11) H. Tanabe, Y. Kobayashi, Y. Matuo, N. Nishi and F. Wada, *Agric. Biol. Chem.*, **48**, 2113 (1984).
12) J. Sugiura, M. Yashuda, S. Kamimiya, K. Izaki and H. Takahashi, *J. Gen. Appl. Microbiol.*, **30**, 167 (1984).
13) H. Tanabe, R. Matsuo and Y. Kobayashi, *Agric. Biol. Chem.*, **49**, 3595 (1985).
14) H. Tanabe and Y. Kobayashi, *Agric. Biol. Chem.*, **50**, 2779 (1986).
15) J. L. Ried and A. Collmer, *Appl. Environ. Microbiol.*, **52**, 305 (1986).
16) J. Sugiura, *Nippon Nōgeikagaku Kaishi*, **62**, 1360 (1988).
17) S. Hasegawa and C. W. Nagel, *J. Food Sci.*, **31**, 838 (1966).
18) C. W. Nagel and T. M. Wilson, *Appl. Microbiol.*, **20**, 374 (1970).
19) B. A. Dave and R. H. Vaughn, *J. Bacteriol.*, **108**, 166 (1971).
20) A. Kaji, I. Fujikawa, S. Iwahara and M. Sato, *Nippon Nōgeikagaku Kaishi*, **46**, 509 (1972).
21) O. P. Ward and W. M. Fogarty, *J. Gen. Microbiol.*, **73**, 439 (1972).
22) A. Chesson and R. C. Codner, *J. Appl. Bacteriol.*, **44**, 347 (1978).
23) H. Tanabe, Y. Kobayashi and I. Akamatsu, *Agric. Biol. Chem.*, **52**, 1855 (1988).
24) M. Sato and A. Kaji, *Agric. Biol. Chem.*, **39**, 819 (1975).
25) M. Sato and A. Kaji, *Agric. Biol. Chem.*, **44**, 1345 (1980).
26) P. Pupillo, *Physiol. Plant Pathol.*, **9**, 113 (1976).
27) J. G. Hancock, *Phytopathology*, **66**, 40 (1976).
28) M. Sato and A. Kaji, *Agric. Biol. Chem.*, **41**, 2193 (1977).
29) C. Hatanaka and J. Ozawa, *Agric. Biol. Chem.*, **36**, 2307 (1972).
30) E. F. Hartree, *Anal. Biochem.*, **48**, 422 (1972).
31) O. Vesterberg, in "Method in Enzymology," Vol. 22, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1971, pp. 389–412.
32) K. Hayashi and Y. Ohba, *Tanpakushitsu Kakusan Koso*, **17**, 304 (1972).
33) W. Diezel, G. Kopperschläger and E. Hofmann, *Anal. Biochem.*, **48**, 617 (1972).
34) S. Y. T. Tam, *J. Food Sci.*, **48**, 532 (1983).
35) P. J. Wood and I. R. Siddiqui, *Anal. Biochem.*, **39**, 418 (1971).
36) J. L. Hedrick and A. J. Smith, *Arch. Biochem. Biophys.*, **126**, 155 (1968).