Isolation and Fundamental Properties of *endo*-Pectate Lyase pI-Isozymes from *Erwinia carotovora*†

Hiroyuki TANABE, Yoshinari KOBAYASHI, Yuhsi MATUO,*
Nozomu NISHI* and Fumio WADA*

Government Industrial Research Institute, Shikoku,
2–3–3, Hananomiya-cho, Takamatsu 761, Japan
**Department of Endocrinology, Kagawa Medical School,*
1750–1, Miki-cho, Kita-gun, Kagawa 761–07, Japan

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A strain of *Erwinia carotovora* was found to produce extracellularly four kinds of *endo*-pectate lyase pI-isozyme (PATE-I, -II, -III and -IV) having similar properties. The four pI-isozymes were purified to homogenous states by ion exchange chromatography, gel filtration and isoelectric focusing. The approximate molecular weights were 28,000 for PATE-I and -III, 32,000 for PATE-II and 33,000 for PATE-IV, respectively, and they had isoelectric points of 10～11. The optimum pHs for the reaction catalyzed by these pI-isozymes were 9.3 for PATE-II, 9.5 for PATE-IV and 9.7 for PATE-I and III, respectively. There were no differences in the optimum Ca^{2+} concentration (0.5～0.6 mM) or *Km* and V_{max} among these four pI-isozymes. The mode of action of all the pI-isozymes was determined to be the *endo*-type.

Biochemical pulping of bast fibers is closely associated with enzymatic maceration of plant tissues in plant pathogenesis.[1] Pectolytic enzymes, especially *endo*-polygalacturonase (EC 3.2.1.15), *endo*-pectin and pectate lyase (EC 4.2.2.10 and EC 4.2.2.2, respectively), are the primary agents responsible for the maceration process.[2～4] Among them *endo*-pectate lyase or polygalacturonate *trans*-eliminase (PATE) produced by bacteria, *e.g.*, *Bacillus*,[5～7] *Xanthomonas*,[8,9] *Pseudomonas*,[10～12] *Clostridium*,[13] *Aeromonas*[14] and *Erwinia*,[15,16] is an alkalophilic enzyme which has been assumed to have higher accessibility to interfiber bonding meterials of bast fibers as the cellulosic fibers are well swollen under alkaline conditions. The authors' screening tests[17] revealed that *Erwinia carotovora*, including *E. aroideae* since the International Code of Nomenclature of Bacteria was revised in 1980, was one of the most promising bacteria for biochemical pulping because of its high producibility of *endo*-PATE as well as its rapid growth.

The present study in this series deals with the purification of enzymes related to *endo*-PATE extracellularly produced by *E. carotovora* and their characterization with the purpose of elucidating the maceration mechanism for bast fibers.

MATERIALS AND METHODS

Reagents. CM-Sepharose CL-6B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals AB. Ampholine carrier ampholytes were obtained from LKB Produkter AB. Marker proteins used were bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000) and ribonuclease (13,700). All other chemicals were of analytical grade and from Wakô Pure Chemical Industries Ltd.

Organism and culture conditions. E. carotovora GIR 726 (FERM P-7579) was originally isolated by the National Institute of Agricultural Science and was kindly supplied by Kagawa Prefecture Agricultural Experimental Station. The basal medium consisted of 5 g/liter of citrus pectin (Sunkist Growers, Inc.), 5 g/liter of monosodium L-

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glutamate, 3 g/liter of $(NH_4)_2SO_4$, 2.4 g/liter of KH_2PO_4, 0.8 g/liter of Na_2HPO_4, 0.5 g/liter of $MgSO_4 \cdot 7H_2O$ and 0.2 g/liter of $CaCl_2$. The initial pH of the medium was adjusted to 7.0 with 2 N-NaOH. The organism was aerobically pre-cultured at 28°C on a rotary shaker for 48 hr. The seed culture (100 ml) was inoculated into a jar fermenter (10 liters) containing 5.0 liters of the medium. The cultivation was carried out at 28°C for 20 hr with aeration at 1.0 liter/min under 400 rpm stirring without pH control. After cultivation, the cells were removed by centrifugation ($5,000 \times g$) for 10 min. the resulting clear supernatant was concentrated and equilibrated with 10 mM sodium phosphate buffer (pH 6.0) using an Amicon membrane filter with molecular weight fractionation of 10,000, and used as the crude enzyme.

Assay of PATE. PATE was assayed according to the modified method of MacMillan and Vaughn[18]: The reaction mixture was composed of 1.0 ml of 0.5% pectate solution adjusted to pH 7.2 with 2 N-NaOH, 0.9 ml of 0.2 M NH_4Cl–NH_4OH buffer (pH 9.5) containing 1 mM $CaCl_2$ and 0.1 ml of the enzyme solution. After incubation at 30°C for 10 min, the enzyme was inactivated by a pH decrease to 4.0 obtained by addition of 2.0 ml of 0.2 M AcOH–AcONa buffer (pH 3.8). One unit of the enzyme was defined as the activity releasing 1 μmol of unsaturated galacturonic acid per min. The amount of the reaction product was determined from the increase in absorbance at 235 nm and then calculated using a factor of 4600 $M^{-1}cm^{-1}$ as the molecular extinction coefficient.[19]

Paper chromatography. Reaction products were examined by descending chromatography on Tôyô Filter Paper No. 51 with a solvent system of AcOEt–pyridine–water–AcOH (5 : 3 : 3 : 2, by volume) with 40 hr development.[20] Spots of unsaturated products were detected with ammoniac $AgNO_3$.

Electrophoresis. Ioelectric focusing was done according to the method of Vesterberg[21] in a sucrose gradient in a column of approximately 110 ml. Isoelectric points were determined by measuring the pH of the fractions at 4°C. Polyacrylamide gel disc electrophoresis was carried out according to the method of Reisfeld *et al.*,[22] using 8.5% gel.

Protein determination. Protein concentrations were determined by the method of Lowry *et al.*[23] or by the measurement of absorbance at 280 nm of fractionated samples in the chromatographic and isoelectric focusing procedures.

Comparison ratio of the rate of increase in fluidity vs. the rate of increase in reducing groups. Viscometric and reductometric assays were performed in parallel at 30°C using a reaction mixture of 2.5 ml of 2.0% pectate solution (pH 7.2), 2.0 ml of 0.2 M NH_4Cl–NH_4OH buffer (pH 9.5) containing 1 mM $CaCl_2$ and 0.5 ml of the purified enzyme solution. The comparison ratio (CR) was computed according to Tam's method.[24]

RESULTS

1. *Purification of PATE*

Step 1: Ion exchange column chromatography. The crude enzyme solution (29 ml) was charged onto a CM-Sepharose CL-6B column, previously equilibrated with 10 mM sodium phosphate buffer (pH 6.0), followed by washing with the same buffer. Elution was carried out with a linear gradient of 0.5 M NaCl in the same buffer (Fig. 1). The active fractions were referred to as "pI-isozymes" of PATE, PATE-I, -II, -III and -IV, on the basis of the elution order. pI-Isozymes were here defined as enzymes with the same activity but different isoelectric points secreted by a microorganism strain.

Step 2: Gelfiltration. The four fractions were separately applied on a Sephacryl S-200 column, previously equilibrated with 0.1 M sodium phosphate buffer (pH 7.5). The results for the PATE-III pI-isozyme is shown as a typical example (Fig. 2). Specific activities of the pI-

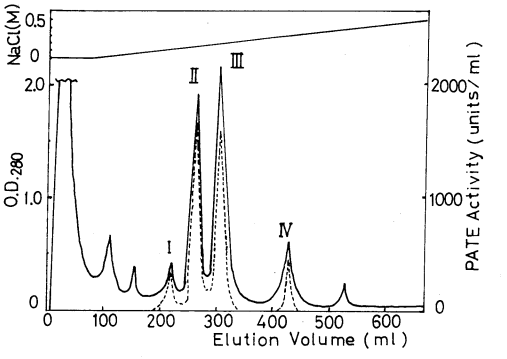

FIG. 1. Chromatography of Crude Enzyme on a CM-Sepharose CL-6B Column.

The crude enzyme (568.4 mg in 29 ml) containing 72,529 units was applied on a column of CM-Sepharose CL-6B (column size: 1.6 cm × 30 cm), previously equilibrated with 10 mM sodium phosphate buffer (pH 6.0). The charged column was developed with a gradient of 0.5 M NaCl in the same buffer at a flow rate of 20 ml/hr. ——, proteins ($OD_{280\ nm}$); ------, PATE activity. Fractionation: I, 200 ~ 216 ml; II, 250 ~ 286 ml; III, 294 ~ 326 ml; IV, 412 ~ 430 ml.

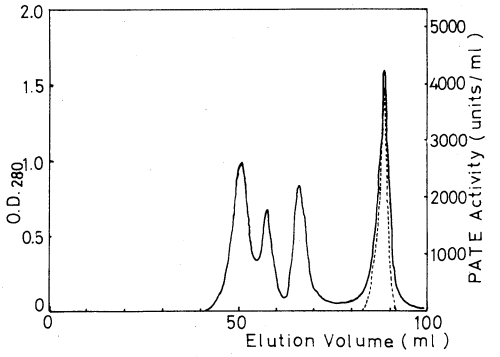

FIG. 2. Gel Filtration of *endo*-PATE pI-Isozyme III on a Sephacryl S-200 Column.

PATE pI-isozyme-III obtained on CM-Sepharose column chromatography (see Fig. 1) was applied on a Sephacryl S-200 column (1.6 cm × 62 cm), previously equilibrated with 0.1 M sodium phosphate buffer (pH 7.5). The column was developed with the same buffer at a flow rate of 10 ml/hr. ——, proteins ($OD_{280\ nm}$); ------, PATE activity.

isozymes were increased about 2 ~ 3 fold by this procedure (Table I).

Step 3: *First isoelectric focusing in wide pH gradients.* Isoelectric focusing of PATE I to IV obtained in step 2 was performed using an Ampholine mixture with a wide pH range of 3.5 to 10. By this procedure, the isoelectric points of the four isozymes were estimated to be higher than 10, and their specific activities were increased 1.8 ~ 3 fold (Table I).

Step 4: *Second isoelectric focusing in narrow pH gradients.* Final purification of the four fractions obtained in step 3 was performed by further isoelectric focusing using an Ampholine mixture with a narrow pH range. The mixture suitable for more effective purification was prepared by mixing pH 9 ~ 11 with pH 3.5 ~ 10 Ampholine at the ratio of 8 to 1. The results are shown in Fig. 3; the approximate isoelectric points were determined to be

TABLE I. PURIFICATION SCHEME FOR *endo*-PATE pI-ISOZYMES

Purification step	Volume (ml)	Protein (mg/ml)	PATE activity (units/ml)	Specific activity (units/mg)	Recovery ratio (%)	Purification (fold)
Crude enzyme	29	19.6	2,501	128	100.0	1.0
1 CM-Sepharose CL-6B					93.1[a]	
PATE-I	16	0.2	260	1,318	(100.0)	10.3
PATE-II	36	0.9	902	1,065	(100.0)	8.4
PATE-III	32	1.2	791	666	(100.0)	5.2
PATE-IV	18	0.3	310	1,244	(100.0)	9.8
2 Sephacryl S-200					86.6[a]	
PATE-I	8	0.2	471	2,358	(90.5)	18.6
PATE-II	12	0.8	2,453	3,164	(91.7)	25.3
PATE-III	14	0.8	1,620	2,107	(89.6)	16.5
PATE-IV	8	0.2	644	3,959	(92.3)	31.0
3 1st isoelectric focusing					71.6[a]	
PATE-I	4	0.1	740	6,540	(71.2)	51.1
PATE-II	10	0.4	2,491	6,361	(76.6)	49.7
PATE-III	10	0.3	1,934	6,607	(76.4)	51.7
PATE-IV	6	0.1	735	7,027	(79.0)	54.9
4 2nd isoelectric focusing					55.9[a]	
PATE-I	4	0.1	625	6,961	(60.2)	54.2
PATE-II	8	0.4	2,469	6,900	(61.1)	53.8
PATE-III	8	0.3	1,909	7,072	(60.4)	55.2
PATE-IV	4	0.1	231	7,296	(59.5)	56.8

[a] Relative recovery ratio at each purification step.

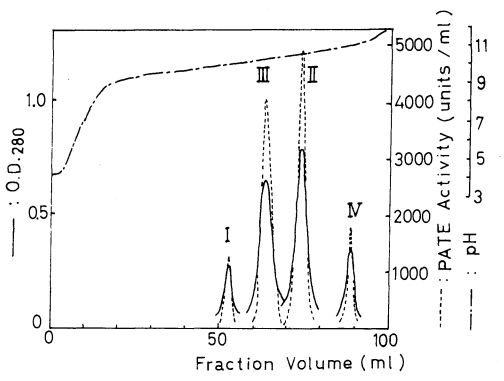

FIG. 3. Final Purification of PATE pI-Isozymes by Isoelectric Focusing with Narrow pH Gradients.

Isoelectric focusing of the *endo*-PATE pI-isozymes was separately conducted using a column of approximately 110 ml with an Ampholine mixture (8 parts of pH 9~11 Ampholine and 1 part of pH 3.5~10 Ampholine) at a constant power of 4.0 W for 40 hr. The final Ampholine concentration was adjusted to be about 1%. The results for the four pI-isozymes are superimposed. I~IV, PATE I~IV.

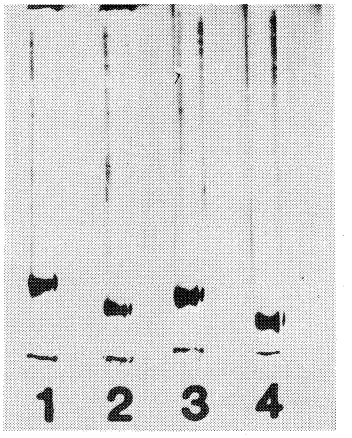

FIG. 4. Polyacrylamide Gel Disc Electrophoresis.

Electrophoresis of the *endo*-PATE pI-isozyme was separately conducted in 8% gel with a stepwise increase of the current of 2.0 to 4.0 mA. Basic fuchsin was used as the tracing marker. 1, PATE-I; 2, PATE-II; 3, PATE-III; 4, PATE-IV.

10.0 for PATE-I, 10.3 for PATE-III, 10.6 for PATE-II and 10.9 for PATE-IV.

The results of the purification are summarized in Table I. pI-Isozymes I to IV were found to be present in the ratio of 1 : 8 : 6 : 1 by calculating the isolated recovery yield. Each pI-isozyme was purified about 54 to 57 fold.

Final step: *Homogeneity test.* The homogeneity of the purified pI-isozymes was examined by polyacrylamide gel disc electrophoresis. All the pI-isozymes gave single bands as shown in Fig. 4.

2. *Properties of purified PATE pI-isozymes*

Molecular weight. The molecular weights of the purified pI-isozymes were estimated to range from 28,000 to 33,000 by the gel filtration method (Fig. 5); the approximate molecular weights were 28,000 for PATE-I and -III, 32,000 for PATE-II and 33,000 for PATE-IV, respectively.

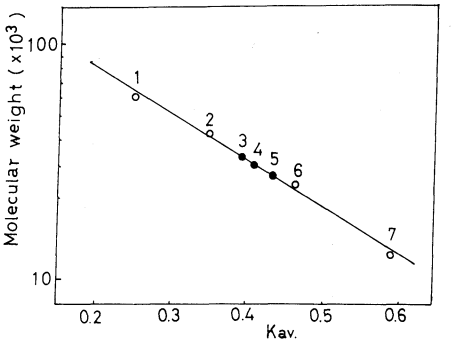

FIG. 5. Estimation of Molecular Weights of *endo*-PATE pI-isozymes by Gel Filtration.

The experimental conditions were the same as those described in the legend to Fig. 2. The slope was obtained by plotting $K_{av.}$ against the log of the molecular weights of the following standard marker proteins: 1, bovine serum albumin (67,000); 2, ovalbumin (43,000); 6, chymotrypsinogen *A* (25,000); 7, ribonuclease (13,700). $K_{av.}$ was calculated as $(V_e - V_0)/V_t - V_0)$, where V_e, V_0 and V_t were the elution volume for each protein, void volume and bed volume, resectively. 3, PATE-IV, 4, PATE-II; 5, PATE-I and -III.

Effect of pH on the enzyme activity. The effects of pH on the activities of the four pI-isozymes are shown in Fig. 6. The optimum pH for the activities of the purified pI-isozymes lay in the limited range of 9.3 to 9.7 regardless of the kind of pI-isozyme; the optimum pHs for the reaction catalyzed by these pI-isozymes were 9.3 for PATE-II, 9.5 for PATE-IV and 9.7 for PATE-I and -III, respectively. No significant change in the pH

optimum was observed for these PATE pI-isozymes in the presence of 0.5 mM Ca^{2+}.

Effect of calcium ions on the enzyme activity. As it was reported that most divalent cations such as Ca^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+} and Mn^{2+} stimulated the activity of PATE from bacteria and fungi,[25] the effect of calcium ions on the PATE pI-isozymes was tested with pectic acid as the substrate. For all pI-isozymes, the activity increased by about 50 to 70% when 0.5 mM Ca^{2+} was added. The activity of the pI-isozymes was also largely dependent on the calcium ion concentration and was found to be optimum in the concentration range of 0.5 to 0.6 mM as shown in Fig. 7.

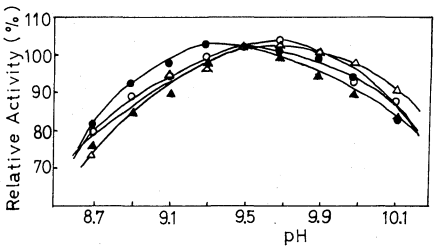

FIG. 6. Effect of pH on Activities of *endo*-PATE pI-Isozymes.

The reaction mixture contained 1.0 ml of 0.5% pectate solution, 0.9 ml of 0.2 M NH_4Cl–NH_4OH buffer with 1 mM $CaCl_2$ and 0.1 ml of the pI-isozyme solution. Activities were expressed as relative values to those at pH 9.5 as a standard. ○, PATE-I; ●, PATE-II; △, PATE-III; ▲, PATE-IV.

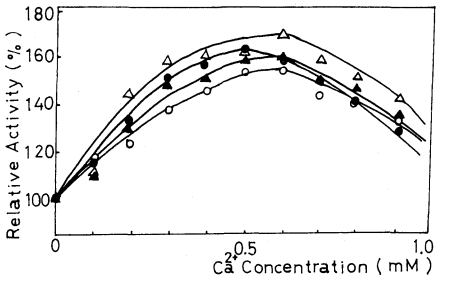

FIG. 7. Effect of Ca^{2+} on Activities of *endo*-PATE pI-Isozymes.

Endo-PATE activity measurements were separately made under the same conditions as described in Fig. 6 except for the varying concentrations of Ca^{2+}. ○, PATE-I; ●, PATE-II; △, PATE-III; ▲, PATE-IV.

Reaction kinetics. The activity of the purified pI-isozymes was measured with various concentrations of pectate in the presence of a fixed calcium concentration (0.5 mM). The results are shown in Fig. 8 as Lineweaver–Burk plots. All the enzymes showed *Km* of 0.050 to 0.055% and V_{max} of 0.69 to 0.80 mmol per mg per min.

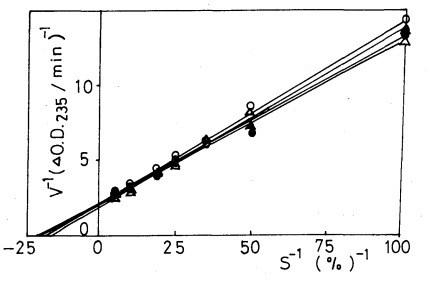

FIG. 8. Lineweaver–Burk Plots for *endo*-PATE pI-Isozymes.

The reaction mixture was composed of 2 ml of pectate solution (pH 7.2) of various concentrations (S%), 2 ml of 0.2 M NH_4Cl–NH_4OH buffer (pH 9.5) containing 1 mM $CaCl_2$ and 2 units of the pI-isozyme. The reaction velocity (V) is represented as the change in absorbance at 235 nm per min. ○, PATE-I; ●, PATE-II; △, PATE-III; ▲, PATE-IV.

TABLE II. FUNDAMENTAL PROPERTIES OF *endo*-PATE pI-ISOZYMES

PATE pI-isozyme	I	II	III	IV
Molecular weight	28,000	31,000	28,000	33,000
Isoelectric point	10.0	10.6	10.3	10.9
Optimum pH	9.7	9.3	9.7	9.5
Optimum Ca^{2+} (mM)	0.6	0.5	0.6	0.6
Km (wt%)	0.054	0.050	0.052	0.055
V_{max} (mmol mg^{-1} min^{-1})	0.70	0.69	0.78	0.80
Comparison ratio[a]	3.77	3.95	3.60	3.90

[a] Comparison ratio = $\dfrac{\Delta \text{ fluidity } (1/\eta_{sp})/\text{time}}{\Delta \mu\text{mol reducing groups/time}}$

All of these properties of the PATE pI-isozymes are summarized in Table II.

Pattern of action. Comparison of changes in absorbancy at 235 nm, paper chromatographic examinations and Tam's method[24] for distiguishing *endo*- from *exo*-polygalacturonases were used to determine the action pattern of PATE pI-isozymes I to IV. The rapid increase in absorbancy and marked decrease in viscosity suggested that the pectate molecule was attacked in a random manner. Chromatograms of the reaction products at comparatively short intervals (10 to 120 min) showed the presence of several unsaturated oligouronides as well as galacturonic acid. The products at 10 min reaction were diverse with at least several components supposedly up to the octamer but merely four products up to tetramer were seen after 2 hr reaction. These results clearly indicated that the pectate molecule was split at inner bonds rather than at terminal ones by all the PATE pI-isozymes. The results were also supported by these with Tam's method.[24] The CR was computed by dividing the rate of increase in fluidity by the rate of increase in reducing groups:

$$\mathrm{CR}=\frac{\Delta\text{fluidity } (1/\eta_{sp})/\text{time}}{\Delta\mu \text{ mol reducing groups/time}}$$

The calculated CR values of PATE pI-isozymes I to IV ranged between 3.60 and 3.95. Tam reported that *exo*-type enzymes came within 0.15 while *endo*-type enzymes lay in the range of 2.04 to 2.99. The CR for the pI-isozymes indicated that they were equally *endo*-types.

DISCUSSION

E. carotovora has been reported to produce PG,[26] *endo*-PATE,[27~29] *exo*-PATE,[30] pectin methyl esterase,[31] cellulase (C_x),[32] phosphatidase,[33] glycerol dehydrogenase[34] and proteinase.[35] The pectinase formation was transformed from that of *endo*-PATE to that of *endo*-pectin *trans*-eliminase (*endo*-PTE) when induced by nalidixic acid.[36,37] Some workers suggested that most of several of these enzymes were involved together in maceration of plant tissue.[3,4] But under alkaline conditions where biochemical pulping proceeded, only small numbers of enzymes such as *endo*- and *exo*-PATE as well as *endo*- and *exo*-PTE were active. Therefore, purification was focused on PATE as a key enzyme for maceration in this study.

It was found that *E. carotovora* FERM P-7579 produced four kinds of *endo*-PATE with extraordinary similarity in properties except for the affinity to CM-Sepharose. Although *E. chrysanthemi* isolate 307, from carnation, produced at least four PATE,[38] there was considerable divergence as to their properties. To the authors' knowledge, few reports have been published showing that *E. carotovora* produces a plural number of PATE.

The pH optima of the four PATE pI-isozymes ranged from 9.3 to 9.7. These values were somewhat higher than the reported data.[39] The molecular weights of the pI-isozymes, 28,000 to 33,000, were almost the same (31,000)[40] or slightly lower (36,000 to 38,000).[39] The PATE pI-isozymes from *E. chrysanthemi* had molecular weights of 30,000 to 36,000 and pH optima between 8.2 to 9.8.[38] Therefore, as far as pH optimum and molecular weight were concerned, the pI-isozymes isolated showed no peculiarity in comparison with those extracellularly secreted by strains of the genus *Erwinia*.

The pI values (10.0 to 10.9) of the pI-isozymes, however, were found to be appreciably higher compared to the values reported for various bacterial PATEs: *e.g.*, the highest pIs ever reported were 9.4 for *E. chrysanthemi*[38] and 10.3 for *Pseudomonas fluorescens*.[12] In spite of the higher pIs, the pI-isozymes were observed to have much difficulty in absorbing to a cationic exchanger at pH 7.0, requiring a decrease in pH to 6.0. In addition, the elution order of the pI-isozymes was not always parallel with the pI rank. This suggested that they would possibly be specific in steric structures as proteins. Furthermore, the higher pIs of the pI-isozymes and the negatively charged substrate

of pectate under alkaline conditions implicated that ionic interactions would be a key to the maceration.

The stimulation of activity by Ca^{2+} was common to the pI-isozymes. This effect was very common to all PATE or PTE originated from bacteria and fungi.

The *Km* values of the PATE pI-isozymes ranged between 0.050 and 0.055%, which were moderate in comparison with those of *Cytophaga johnsonii* (0.22%),[41] *Cl. multiferans* (0.114%),[42] *Ps. fluorescens* (0.005%)[10] and *B. polymixa* (0.121%).[6]

In order to determine the action pattern, Tam's method[24] for distinguishing *endo-* from *exo*-PGs was firstly applied to PATE groups in this study. The CRs computed for the pI-isozymes were found to be very different from those for PGs, which showed that this method was also simple and accurate in determining the mode of PATE attack.

In conclusion, the four pI-isozymes from *E. carotovora* FERM P-7579 were almost completely the same in properties, especially in activities, except for the affinity to a cationic ion exchanger. A plausible explanation proposed was that these pI-isozymes were composed of common active centers with slightly modified inactive parts which caused the affinity difference. Further investigation of this aspect in connnection with the maceration mechanism is in progress.

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