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Extracellular and Intracellular Exopectate Lyases of *Erwinia carotovora* subsp. *carotovora*

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(Figs. 1–7, Tables 1–2)

Erwinia carotovora, one of the most common soft-rot bacterial pathogens, produces several active pectic enzymes, particularly those having a random-action pattern. The activities of endopectate lyase (EC. 4.2.2.2)[1,2] and endo-D-galacturonanase (EC. 3.2.1.15)[3] have both been demonstrated in this pathogen. In addition, a third endoenzyme, pectin lyase (EC. 4.2.2.10), is likely to be the principal constituent of the pectic activities of the pathogen. In fact a high activity of pectin lyase has been observed in the culture fluid of a strain of *E. aroideae*[4], which has been considered to belong to the same species as *E. carotovora*[5]. Among these endoenzymes, the endopectate lyase and the endo-D-galacturonanase degrade pectic acid more rapidly than highly esterified pectinic acid, whereas the pectin lyase is inactive towards pectic acid. Besides these endoenzymes, *Erwinia* has been shown to produce several other exoenzymes, such as oligo-D-galacturonate lyase (EC. 4.2.2.6)[6,7], exopectate lyase (EC. 4.2.2.9)[8], and D-galacturonandigalacturono hydrolase (EC. 3.2.1.82)[9]. All these exoenzymes degrade preferentially pectic acid.

In the culture fluid or in the cells of *Erwinia*, the amount of exoenzymes is usually rather smaller than that of the endoenzymes. Consequently, various side problems arise in the isolation of any of the exoenzymes from the crude enzyme preparations. In this respect, strain W2, which was isolated from sewage and classified in the genous *Erwinia*[10], is a better source of exoenzyme than the other strains of *Erwina* because W2 produces an exopectate lyase (exo-PAL) as the sole pectic enzyme when grown on pectate-bonito extract media[10]. The exo-PAL is capable of liberating 4,5-unsaturated digalacturonic acid from the reducing end of pectic acid molecules. W2 has recently been identified by SAKANE *et al.*[11] as *Erwinia carotovora* subsp. *carotovora*.

The previous report[10] from our group has dealt with the purification of the exo-PAL produced extracellularly in the culture fluid of W2. Although an over-all purification of 264-fold was achieved by a three-step chromatography on Amberlite IRC-50 columns, the recovery of the enzyme activity was only 2.6 % of the activity present in the culture fluid. The present paper deals with the purification and the properties of the extracellular exo-PAL and of another similar exo-PAL present in the cells of the same

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strain of *E.carotovora*. Both enzymes could be purified in moderate yield; their specific activities were comparable to that of the purified preparation in the previous study[10].

MATERIALS AND METHODS

1. Culture methods

Erwinia carotovora subsp. *carotovora*, strain W2, was precultured on 2 % agar slants, pH 7.2, containing 0.5 % peptone, 0.1 % KH_2PO_4 and 0.5 % $Na_2HPO_4 \cdot 12H_2O$ in potato extract for about one day at 30°C. For the extracellular exo-PAL, one loop of the agar slant culture was inoculated into 200 m*l* of a medium in 500-m*l* ROUX flasks and incubated stationarily for 14 days at 30°C. The medium was composed of 0.5 % peptone, 0.5 % oligogalacturonate, 1 % $Na_2HPO_4 \cdot 12H_2O$ and 0.3 % bonito extract; the pH was adjusted to 7.2. For the intracellular exo-PAL, the culture was agitated by a reciprocal shaker (120 strokes per min) for about one day at 30°C; 500-m*l* SAKAGUCHI flasks containing 200 m*l* of the same medium described above were used.

2. Preparation of substrates

Pectic acid was prepared from a commercial preparation of citrus pectinic acid (Ishizu Seiyaku Co., Ltd.) as described previously[12]; the pectinic acid was saponified for 90 min at about 0°C in 0.05 *N* NaOH. Under such conditions, cleavage of the glycosidic bonds of pectinic acid by transelimination was almost depressed[12].

Oligogalacturonate with an average chain length of 5.5 was produced from the citrus pectic acid with a purified endo-D-galacturonanase of *Saccharomyces fragilis* as described previously[13]. This was used as substrate for the assay of the exo-PAL activity and as carbon source for the cultivation of the organism. As substrate, polygalacturonate with an average chain length of 38.9 was also prepared in the same manner except that the pectic acid was degraded by the *Saccharomyces* enzyme to the extent that white powdery precipitates were formed on addition of one volume of 0.2 *N* HCl.

3. Assay of enzyme activity

One unit of the exo-PAL is the amount of enzyme that releases one μmol of 4,5-unsaturated digalacturonic acid from substrates per min at 35°C. Unless stated otherwise, the enzyme activity was always assayed by measuring the increase in absorbance at 235 nm in reaction mixtures. The release of one μmol of 4,5-unsaturated digalacturonic acid per m*l* of the reaction mixture is equivalent to an increase of 4.7 in the absorbance at 235 nm[14].

In the cases where the effects on the enzyme activity of metal ions or ethylenediaminetetraacetate (EDTA) were determined, *N*-ethylmorphorine-HCl buffer was used. In other cases, Tris-HCl or borate buffer also was used.

4. Analytical methods

The average chain lengths of oligogalacturonate and polygalacturonate were obtained from the ratio of the amounts of total galacturonic acid to those of reducing galacturonic acid at the chain ends; these amounts were determined by the carbazole method[15] and by the micro WILLSTÄTTER-SCHUDEL method[16], respectively.

Protein was determined by the FOLIN-LOWRY method, as modified by HAGIHARA[17]. In gel filtration and cellulose ionexchanger chromatographies, the column eluates were continuously monitored at 280 nm with a Nihonbunko Uvidec-100 detector.

RUSULTS

1. Purification of the extracellular exo-PAL

Salting out with ammonium sulfate. The cell-free culture fluid (4*l*) from 14-day-old cultures of the *Erwinia* was dialyzed against 0.01 *M* phosphate buffer, Na_2HPO_4-KH_2PO_4, pH 7.0, for three days. The dialyzed solution was concentrated to about one-tenth of its original volume at 35°C under reduced pressure with a rotary vacuum evaporator and treated with solid ammonium sulfate at 0.6 saturation. After centrifugation at 6,000 x g for 10 min, the precipitates were dissolved in 60 m*l* of deionized water and insoluble materials, if present, were removed by recentrifugation.

Gel filtration chromatography. Ten-m*l* samples were passed through a column (2.5 x 40 cm) of Ultrogel AcA 34 (fractionating range: 20,000 – 400, 000 MW, LKB-Producter AB) previously equilibrated with 0.02 *M* phosphate buffer, pH 7.0. Although most of the colloidal impurities voided the column, appreciable amounts of colored impurities were eluted together with the exo-PAL (Fig. 1). Fractions 15–20 having the activity of the exo-PAL were combined.

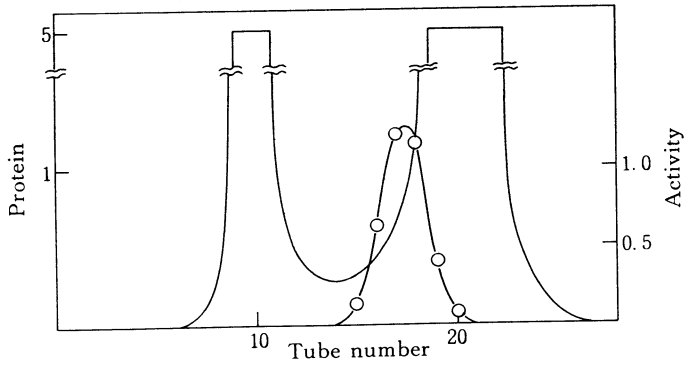

Fig. 1. Gel filtration chromatography on Ultrogel AcA 34 of the fraction salted out with ammonium sulfate.
Gel filtration: flow rate, 50 m*l* per hr; tube volume, 10 m*l*. Assay conditions: sodium polygalacturonate,0.2 %; eluate, 0.1 m*l*; 0.05 *M* Tris-HCl buffer (pH 8.5 at 35°C), 0.3 m*l*; total volume 0.5 m*l*; incubation, for 30 min at 35°C.
——, protein, absorbance at 280 nm; – ○ –, activity, absorbance at 235 nm (x 1/7).

First DEAE-cellulose column chromatography. The eluate (180 m*l*) in the gel filtration chromatography was added to a column (3 x 15 cm) of DEAE-cellulose (0.87 meq/g, Brown Co.) prepared in the same manner as above. The exo-PAL was passed through the column by washing with the same buffer, whereas almost all the colored impurities were adsorbed. The effluent was dialyzed against 0.02 *M* phosphate buffer, pH 6.0, for two days.

P-cellulose column chromatography. The dialyzed solution (280 m*l*) was added to a column (3 x 15 cm) of P-cellulose (1.10 meq/g, Brown Co.) equilibrated with 0.02 *M* phosphate buffer, pH 6.0. The column was washed with the same buffer until the effluent gave almost no absorbance at 280 nm, then the exo-PAL was eluted by a convex gradient elution technique, with a constant volume mixing bottle containing 350 m*l* of 0.02 *M* phosphate buffer, pH 6.0, and the reservoir filled with 1 *M* phosphate buffer, pH 6.0. The effluent passed through the column contained fairly large amounts of proteins but it showed only a feeble activity of the exo-PAL; this fraction was named tentatively enzyme 1-A. Most of the activity was recovered in the fractions 43–52 at the first peak obtained with the gradient elution (Fig. 2). The active fractions were

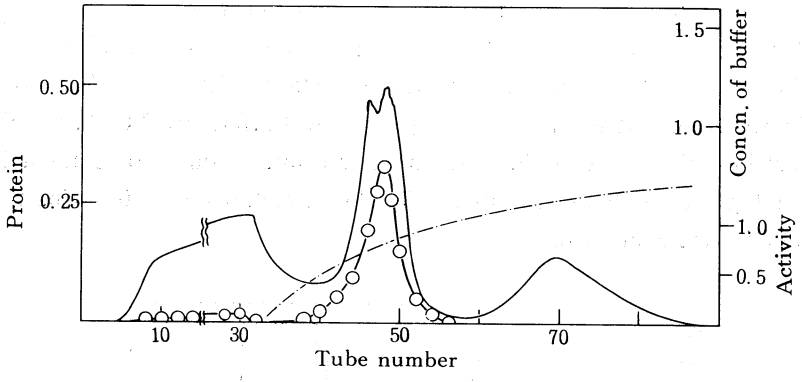

Fig. 2. P-cellulose chromatography of the first DEAE-cellulose effluent. Chromatography: flow rate, about 100 m*l* per hr; tube volume, 10 m*l*.
——, protein, absorbance at 280 nm; –·–·–, concentration of buffer (*M*); – ○ –, activity, absorbance at 235 nm (x 1/7). Other details are as described in Fig. 1.

combined and dialyzed against 0.05 *M* Tris-HCl buffer (pH 9.0 at 15°C) containing 0.02 *M* sodium chloride prior to the next treatment.

Second DEAE-cellulose column chromatography. The dialyzed solution (100 m*l*) was applied to a DEAE-cellulose colum (2 x 25 cm)equilibrated with the same Tris-HCl buffer containing 0.02 *M* sodium chloride. After washing out the unadsorbed proteins with the same buffer, the exo-PAL was eluted with a convex gradient consisting of 350 m*l* of the same Tris-HCl buffer containing 0.02 *M* sodium chloride in the constant volume mixing bottle, and the same Tris-HCl buffer containing 0.2 *M* sodium chloride in the reservoir. As shown in Fig. 3, the activity of the exo-PAL appeared in fractions 23–30. The fractions 24–28 were combined and, after dialysis against 0.02 *M* phosphate buffer of pH 7.0, used as the purified extracellular exo-PAL, which was named tentatively enzyme 1-B. At this final stage, the exo-PAL was usually purified about 270-fold compared with the crude culture fluid (Table 1).

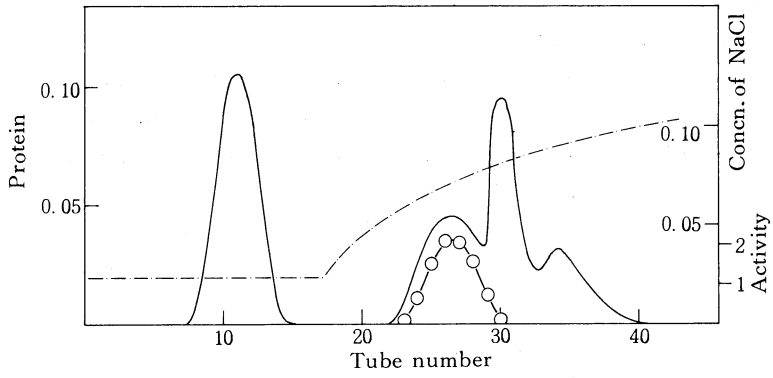

Fig. 3. Second DEAE-cellulose chromatography of the P-cellulose eluate. –·–·–, concentration of NaCl (*M*). Other details are as described in Fig. 2.

Table 1. Summary of purification of the extracellular exo-PAL.

Purification step	Specific activity (unit/mg. X 10^{-2})	Purification (fold)	Recovery (%)
Culture fluid	3.2	1	100
Ammonium sulfate precipitate	9.4	2.9	92.7
Ultrogel AcA filtrate	39.1	12.2	76.9
First DEAE-cellulose effluent	110.8	34.6	66.2
P-cellulose effluent[a)]	46.3	14.5	4.9
P-cellulose eluate	249.9	78.1	28.6
Second DEAE-cellulose eluate[b)]	854.1	266.9	21.6

a) enzyme 1-A

b) enzyme 1-B

Assay conditions: substrate, ammonium polygalacturonate, 0.2%; buffer, 0.05 *M* Tris-HCl (pH 8.5 at 35°C); enzyme, suitable amounts; incubation, for 30 min at 35°C.

2. Purification of the intracellular exo-PAL

Autolysis. After cultivation for about one day, the cells were harvested by centrifugation at 6,000 x g for 10 min and washed with deionized water. The washed cells (about 40 g per 4 *l* of the culture medium) were suspended in 10 parts of 0.02 *M* phosphate buffer, pH 7.0, containing 2 % toluene and stirred for 30 min at 30°C, then the contents were allowed to stand overnight at 30°C. The autolyzate was cleared of cell debris by centrifugation at 6,000 x g for 10 min. After dialysis against 0.02 *M* phosphate buffer, pH 7.0, overnight, the supernatant solution was used as the crude enzyme solution of the intracellular exo-PAL.

Salting out with ammonium sulfate. The fraction precipitated at 0.3–0.8 saturation with ammonium sulfate from the dialyzed autolyzate was collected by centrifugation at

6,000 x g for 10 min. The precipitate was dissolved in 50 m*l* of 0.04 *M* phosphate buffer, pH 8.0, and dialyzed against the same buffer.

First DEAE-cellulose column chromatography. The above dialyzed fraction was added to a DEAE-cellulose column (2 x 28 cm) equilibrated previously with 0.04 *M* phosphate buffer, pH 8.0. Most of the activity of the exo-PAL appeared in the effluent from the column with the same buffer. The effluent (about 130 m*l*) was concentrated to about 60 m*l* by pervaporation at room temperature. The concentrated solution was dialyzed against 0.05 *M* Tris-HCl buffer (pH 9.0 at 15°C) containing 0.02 *M* sodium chloride prior to the next treatment.

Second DEAE-cellulose column chromatography. The dialyzed solution was rechromatographed on a column (2 x 28 cm) of DEAE-cellulose equilibrated with the same Tris-HCl buffer. After washing with the same buffer until the effluent contained only traces of proteins, elution was carried out with a convex gradient of sodium chloride in the buffer; the concentration of sodium chloride was 0.02 *M* and 0.2 *M* in the constant volume mixing bottle (350 m*l*) and in the reservoir, respectively. As shown in Fig. 4, the

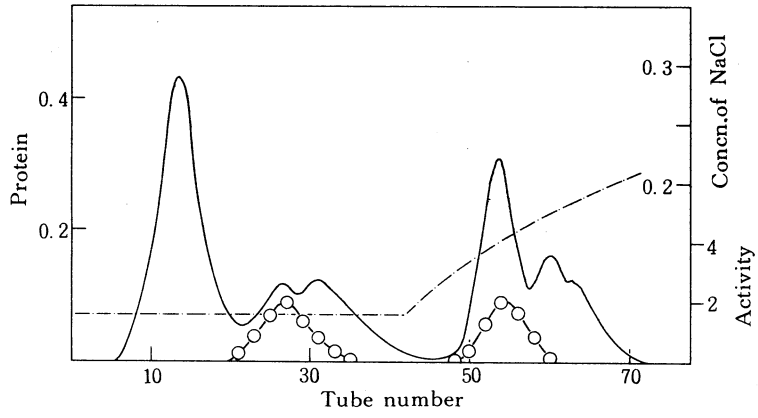

Fig. 4. Second DEAE-cellulose chromatography of the first DEAE-cellulose effluent.
Details are as described in Fig. 3.

exo-PAL activity fell into two peaks; under the condition of sodium chloride at 0.02 *M*, one passed through the column, while the other remained on the column. Fractions 21–32 and fractions 49–58 were pooled separately and, after dialysis against 0.02 *M* phosphate buffer of pH 7.0, used as the purified intracellular exo-PALs. These two preparations were tentatively named enzyme 2-A and 2-B in their order of elution. This purification resulted in 65- and 30-fold over-all purifications in the specific activities of enzyme 2-A and 2-B, respectively. The total recovery of the two enzymes was about 35 % (Table 2).

Table 2. Summary of purification of the intracellular exo-PAL.

Purification step	Specific activity (unit/mg, x 10^{-2})	Purification (fold)	Recovery (%)
Crude enzyme solution	8.2	1	100
Ammonium sulfate precipitate	113.7	13.9	78.1
First DEAE-cellulose effluent	176.6	21.5	57.1
Second DEAE-cellulose effluent[a)]	534.2	65.1	21.4
Second DEAE-cellulose eluate[b)]	245.5	29.9	14.0

a) enzyme 2-A
b) enzyme 2-B
Details are as described in Table 1.

3. Properties of the exo-PALs

Optimum pH. The effect of pH on the activities of enzyme 1-B, 2-A, and 2-B was determined at various pH values with *N*-ethylmorphorine-HCl and borate buffers. The results in Fig. 5 show that each enzyme has a similar optimum pH, which lies on a rather wide range between pH 8.5–9.5. At the alkaline side above pH8.0, almost no difference

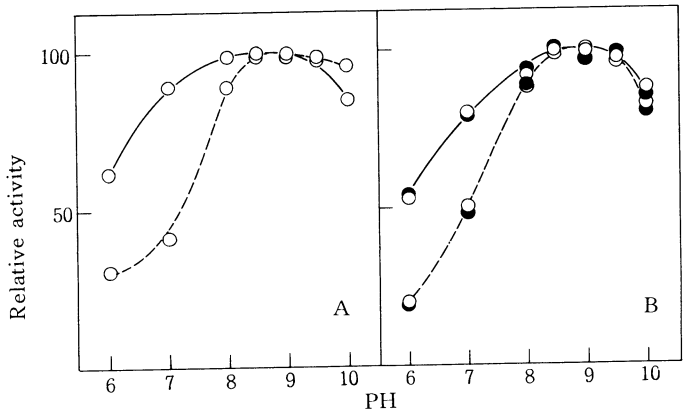

Fig. 5. Effect of pH on the exo-PAL activities.
Assay conditions: substrate, 1 % ammonium polygalacturonate, 0.1 m*l*; buffer, 0.3 m*l*; enzyme, about 7 x 10^{-3} unit, 0.1 m*l*; incubation, for 30 min at 35°C. The enzyme solution was dialyzed previously against 0.005 *M* ammonium phosphate buffer, pH 7.0, containing 75 m*M* NaCl.
(A), enzyme 1-B; (B), enzyme 2-A (–○–), enzyme 2-B (–●–); ——, borate buffer, pH 6–9.5 (0.1 *M* KH_2PO_4-0.05 *M* $K_2B_4O_7$), pH 10 (0.1 *M* KOH-0.05 *M* $K_2B_4O_7$); - - - - -, 0.2*M N*-ethylmorphorine-HCl buffer.

in their activities was found between the two buffers, but, below pH 8.0, the activities in the ethylmorphorine-HCl buffer were much lower than those in the borate buffer.

Effect of sodium chloride. The effects of varying concentration of sodium chloride on the exo-PAL activities are shown in Fig. 6. Maximum activation occurred at about 15

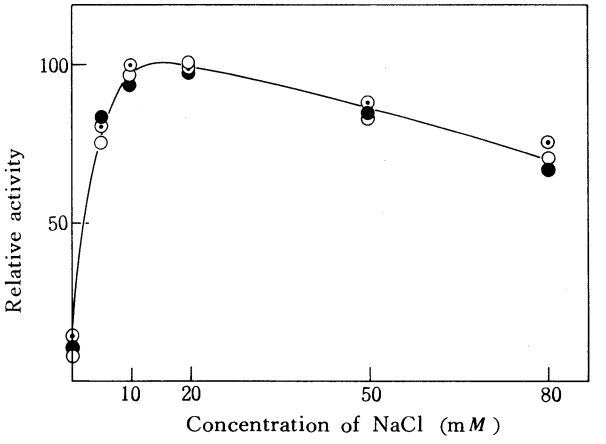

Fig. 6. Effect of sodium chloride on the exo-PAL activities. Assay conditions: substrate, ammonium polygalacturonate, 0.2 %; buffer, 0.04*M* *N*-ethylmorphorine-HCl, pH 9.0; enzyme, about 7 x 10^{-3} unit per m*l* of reaction mixture; incubation, for 30 min at 35°C. -○-, enzyme 1-B; -⊙-, enzyme 2-A; -●-, enzyme 2-B.

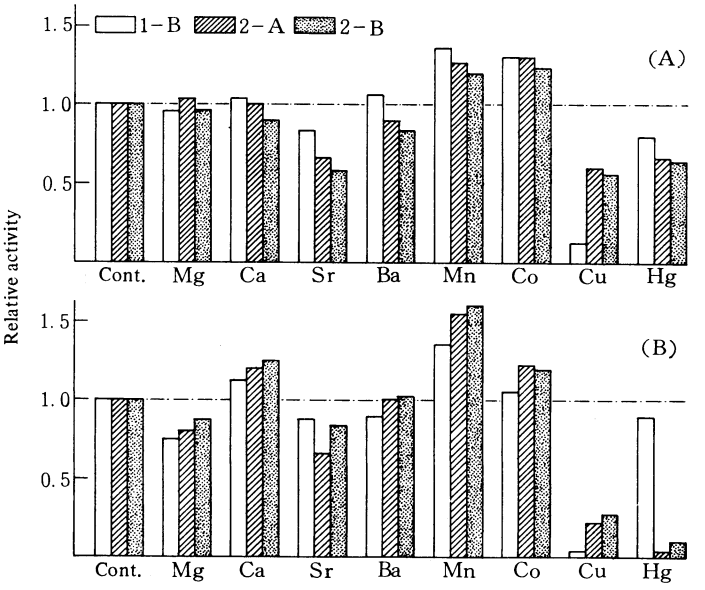

Fig. 7. Effect of divalent cations on the exo-PAL activities in the presence or absence of sodium chloride.
Assay conditions: substrate, 5m*M* oligogalcturonate (*N*-ethylmorphorine-salt); buffer, 0.04 *M* *N*-thylmorphorine-HCl, pH 9.0; divalent cation, 1 m*M*; enzyme, about 7 x 10^{-3} unit of enzyme 1-B, 2-A, or 2-B per m*l* of reaction mixture; incubation, for 30 min at 35°C. (A), presence of 15 m*M* NaCl; (B), absence of NaCl.

m*M* sodium chloride, but the activities decreased gradually at concentrations higher than 15 m*M*.

Effects of divalent cations. Fig. 7-A and 7-B show the effects of divalent cations in the presence and in the absence of 15 m*M* sodium chloride, respectively. In the absence of sodium chloride, Mn^{2+} showed a stimulatory effect on the exo-PAL activities; Ca^{2+} and Co^{2+} also produced an activation, although they were not so effective as Mn^{2+}. In the presence of sodium chloride, Ca^{2+} showed no stimulation, but the activation by Co^{2+} was almost the same as that by Mn^{2+}. In the absence of sodium chloride, the activities of enzyme 2-A and 2-B were markedly inhibited by Cu^{2+} and Hg^{2+}, but their inhibitory effects were weakened to a certain extent in the presence of sodium chloride. In the case of the enzyme 1-B, regardless of the presence or absence of sodium chloride, Cu^{2+} was a strong inhibitor, but the inhibition by Hg^{2+} was not so strong. Sr^{2+}, Mg^{2+}, and Ba^{2+} produced also some inhibitory effects.

Inhibitory effect of EDTA. Addition of 1 m*M* EDTA to the reaction mixtures resulted in a complete loss of all the three enzyme activities.

DISCUSSION

In a strain of *E.carotovora*, an exo-PAL activity was found both in the culture fluid and in the cells. By use of cellulose ionexchangers, each activity was separated into two fractions; they were named tentatively enzyme 1-A and 1-B for the extracellular exo-PALs, and enzyme 2-A and 2-B for the intracellular exo-PALs. Since the activity of enzyme 1-A was very low, the properties of the other three enzymes were investigated. Under the conditions in this experiment, the properties of the extracellular exo-PAL, 1-B, were found to be comparable in every respect, except Hg^{2+}-inhibition, to those of the intracellular exo-PALs, 2-A and 2-B. This indicates that the extracellular and intracellular exo-PALs are probably identical, and that both the intracellular exo-PALs, 2-A and 2-B, might be regarded as the same enzyme. The yields of enzyme 1-B, 2-A, and 2-B were 21.6, 21.4, and 14.0 %, respectively; the specific activity of each enzyme was comparable to that of the purified exo-PAL reported previously[10]. As the enzyme source, the intracellular exo-PAL is superior to the extracellular enzyme because the total yield of the former was higher than that of the latter and, moreover, the former can be purified more easily than the latter.

The high optimum pH, about 9, of the exo-PAL of our *Erwinia* is in good agreement with those reported for the other bacterial pectate lyases; namely, the pH optima reported either for the endopectate lyases of *Bacillus polymyxa*[18], *E.carotovora*[1], *E. aroideae*[8], and *Pseudomonas fluorescens*[19] or for the exopectate lyases of *E. aroideae*, *E.dissolvens*[20], and *Clostridium multifermentans*[21] lie in the pH range of 8.0–9.5.

Another characteristic property of the bacterial pectate lyases is the absolute requirement for Ca^{2+} or some other divalent cations in order to maintain their optimal activities. All the above pectate lyases except the exo-PALs of *Erwinia* are markedly activated by Ca^{2+}. In the present experiment, Ca^{2+} also stimulated the activity of the exo-PAL.

However, its stimulatory effect was exerted only in the absence of sodium chloride and, even in this case, was not so effective; Ca^{2+} had no effect in the presence of sodium chloride. The exo-PAL was also stimulated by Mn^{2+} and Co^{2+}, but they were not so effective either. Of the cations tested in this experiment, Na^{+} was the most effective activator. When ammonium polygalacturonate was used as substrate, the maximum activation was obtained with sodium chloride at about 15 m*M*. Similar results have already been reported in the previous paper[21], in which we thought that the exo-PAL of the *Erwinia* had an absolute requirement for Na^{+}. However, the mechanism of stimulation by Na^{+} has remained obscure. Na^{+} might closely be related in chemical structure of the substrate, ammonium polygalacturonate, in the aqueous solutions because replacement of the ammonium polygalacturonate by an oligogalacturonate as substrate caused a considerable decrease in the stimulatory effect of Na^{+} [22].

In general, complete depression of an enzyme activity by the addition of EDTA is regarded as one of the strongest pieces of evidence in support of the absolute requirement of certain divalent cations for the enzyme activity. The activity of the exo-PAL of our *Erwinia* was completely depressed by the addition of 1 m*M* EDTA. As reported in the previous paper[21], in the presence of sodium chloride, the activity was restored considerably by the addition of divalent cations, such as Ca^{2+}, Mg^{2+}, Co^{2+}, and Mn^{2+}. These results might be taken to indicate that this enzyme also has an absolute requirement for some divalent cations. However, we recently ascertained that some chelating agents other than EDTA do not have any inhibitory effects and behave even as an activator. The details of the inhibitory effect of EDTA are now under investigation and will be reported in the near future.

SUMMARY

A strain of *Erwinia carotovora* was found to produce an exopectate lyase (exo-PAL) both in the culture fluid and in the cells. Each activity of the extracellular and intracellular exo-PALs fell into two fractions by cellulose ionexchanger chromatographies. They were named tentatively enzyme 1-A and 1-B, and 2-A and 2-B, for the former and the latter, respectively. As the amount of enzyme 1-A was very small, it was discarded. The yields of enzyme 1-B, 2-A, and 2-B were 21.6, 21.4, and 14.0 %, respectively; the purification degree of each enzyme was considerably high. These three exo-PALs resembled one another very closely in the following respects: (1) The optimum pH was about 9 in both Tris-HCl and borate buffers. (2) Na^{+} was an effective activator; its optimum concentration was about 15 m*M*. Mn^{2+} and Co^{2+} stimulated weakly regardless of the presence or absence of Na^{+}, but Ca^{2+} showed a weak stimulation only in the absence of Na^{+}. (3) The exo-PALs were inhibited to varying extent by Cu^{2+}, Hg^{2+}, Sr^{2+}, Mg^{2+}, and Ba^{2+}. (4) Addition of 1 m*M* EDTA led to a total loss of activity. From these results, we considered that both the extracellular and intracellular exo-PALs are probably identical. As enzyme source, the intracellular exo-PAL is superior to the extracellular exo-PAL because of the high recovery and ease of preparation of the former enzyme.

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Erwinia carotovora subsp. *carotovora* の菌体外及び菌体内エキソペクテートリアーゼ

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E. carotovora は代表的な軟腐病菌であり，強力なエンドペクチナーゼを生産することで知られている。本研究で用いたW2株も *E. carotovora* に属することがわかっている。しかしW2株は通常株と異なり，エンド酵素をつくらず，エキソペクテートリアーゼ(exo- PAL)のみを生産するので理想的な exo- PAL 給源である。前報では exo- PAL を菌体外酵素として分離したが，最近，この酵素が菌体内にも多量に生産されることを知った。両酵素がかなりな純度にまで精製されたので，ここでは精製法と2，3の性質について報告した。なお，両酵素はセルロースイオン交換体で処理すると，それぞれ二つの画分に分かれたので，仮に菌体外酵素を1－A，1－B，菌体内酵素を2－A，2－Bと呼ぶことにした。ただし1－Aについては量的にわずかであったので検討しなかった。各酵素の回収率は1－B 21.6％，2－A 21.4％及び2－B 14.0％であった。

3種類の酵素の性質は調査範囲内では非常によく一致した。すなわち，(1)最適 pH は9付近である。(2) Na^+(15mM)で強く活性化される。Mn^{2+} と Co^{2+} はNa^+の有無にかかわらず弱い促進効果を示すが,Ca^{2+}はNa^+無添加でのみ弱い促進効果を示す。(3) Cu^{2+}，Hg^{2+}，Sr^{2+}，Mg^{2+} 及び Ba^{2+} は程度は異なるが，いずれも阻害作用を示す。(4) EDTA (1mM)の添加で完全に失活する。

以上の結果は菌体外酵素と菌体内酵素が同一酵素であることを示しているものと思われる。なお酵素給源としては，菌体内酵素の方が精製も容易であり，総回収率も高いので有利である。