Purification and Properties of Pectin *trans*-Eliminase from *Aspergillus sojae*

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Pectin transeliminase from the culture medium of *Aspergillus sojae* was purified 940-fold by ammonium sulfate fractionation, column chromatography on CM-Cellulose, DEAE-Sephadex and SE-Sephadex and gel filtration. The purified enzyme was free from polygalacturonase, hemicellulase, cellulase and protease, and was almost homogeneous on disc electrophoresis. Using gel filtration a molecular weight of about 32,000 was estimated for the enzyme. The pH optimum of the enzyme for pectin (68% esterified) was 5.5, but was 7.0 for polymethyl polygalacturonate methyl glycoside (98% esterified). The addition of divalent cation to pectin stimulated the activity and shifted the pH optimum to neutral side. This tendency by divalent cation, however, was not observed when polymethyl polygalacturonate methyl glycoside was used as substrate. The enzyme is stable between pH 4 to 7. Heating the enzyme solution at 70°C for 10 min caused complete loss of activity. A marked inhibition of activity was produced by oxidizing reagents such as iodine and N-bromosuccinimide. In contrast, no effect emerged from the action of chelating agents, reducing reagents and those reagents which convert SH-groups in protein into mercaptides. The limits of degradation of pectin and polymethyl polygalacturonate methyl glycoside by the enzyme were 26.3 and 39.8%, respectively.

In 1960 Albersheim *et al.*¹⁾ reported an enzyme which degraded α -1, 4 galacturonide linkages in pectin by a trans-elimination mechanism. The enzyme, namely, pectin transeliminase (PTE) (E.C. 4.2.99) is specific for methyl-esterified galacturonide linkage.

Similar enzymes (pectic acid transeliminase), which are specific for non-esterified galacturonide linkage, have been reported to be found in bacteria.^{2~5)} However, the properties of PTE have not been known in detail, because only a few reports have been published so far.^{6~8)}

In the course of an investigation of the enzymatic degradation of plant cell walls by *Aspergillus sojae*, we found that the fungus produced PTE extracellularly in significant amounts,⁹⁾ and the enzyme was able to macerate various kinds of plant tissue by itself.¹⁰⁾

PTE of this fungus was also found to be able to clarify the cloudy fruit juice such as those of apple and grape.¹¹⁾

The present paper describes the purification and properties of PTE from *Asp. sojae*.

MATERIALS AND METHODS

Pectic substances. Pectin was obtained from Sunkist Growers, Inc. It was washed three times with 70% (v/v) ethanol to remove soluble sugars. Polymethyl polygalacturonate methyl glycoside (Link pectin) was prepared by the method of Morell *et al.*¹²⁾ The degrees of esterification of pectin and Link pectin were 68 and 98%, respectively.

Cultivation of *Aspergillus sojae*. *Asp. sojae* No. 48 was grown on 400 g of wheat bran moistened with 320 ml of water in wooden boxes (60×30×5 cm) at 30°C for 3 days.

Assay of PTE activity. PTE was determined by spectrophotometry. Reaction mixtures containing 0.5 ml of enzyme solution, 1.0 ml of 1% pectin and 1.0 ml of McIlvaine buffer, pH 5.5, were incubated at 40°C for a definite period. Increase in an absorbance at 235 m μ was measured using a Hitachi Model 101 spectrophotometer. One unit of PTE was defined as the increase of absorbance at 235 m μ by 1.0 in the reaction mixture per min.

Protein concentration. Protein concentrations were determined by the method of Lowry *et al.*¹³⁾ by using bovine serum albumin as a standard.

RESULTS

Purification of enzyme

All procedures were carried out at 0~4°C except otherwise stated.

Step 1. Extraction. To 8 kg of bran culture of *Asp. sojae* No. 48 were added 40 liters of water. The suspension was stirred at room temperature for 2 hr, and filtered with cotton cloth. Clear extract (34 liters) was obtained by further filtration with celite.

Step 2. Ammonium sulfate fractionation. Solid ammonium sulfate (10.2 kg) was added to clear extract with continuous stirring. After standing for 4 hr, the precipitates formed were removed by centrifugation at 8000 $\times g$ for 10 min. To the supernatant ammonium sulfate (9.2 kg) was added with stirring. After standing overnight, the precipitates formed were collected by centrifugation at 8000 $\times g$ for 10 min, and dissolved in distilled water. Undissolved materials were removed by centrifugation at 10,000 $\times g$ for 10 min. Each 50-ml portion of the solution was passed through a Sephadex G-25 column (2 $\times$ 140 cm) to remove ammonium sulfate. Active fractions free from salts were collected and lyophilized.

Step 3. Batchwise treatment with DEAE-Sephadex. The lyophilized powder (60.4 g) was dissolved in 0.01 M acetate buffer, pH 4.0, and undissolved materials were removed by

centrifugation at 8000 $\times g$ for 10 min. The solution was treated with DEAE-Sephadex previously equilibrated with 0.01 M acetate buffer, pH 4.0 in a batchwise. Most of cellulase, hemicellulase and colored materials were removed by this treatment.

Step 4. CM-Cellulose column chromatography. The treated solution was applied on a CM-cellulose column (3 $\times$ 55 cm) previously equilibrated with 0.01 M acetate buffer, pH 4.0. About 40% of the activity were passed through the column. After washing with 0.01 M acetate buffer, pH 4.0, the column was eluted successively with 0.05 M acetate buffer, pH 4.5 and 0.1 M acetate buffer, pH 5.0. Adsorbed activity was eluted from CM-cellulose column with 0.1 M acetate buffer, pH 5.0, and active fractions (200 ml) were collected and lyophilized.

Step 5. DEAE-Sephadex column chromatography. The powder obtained in the above step was dissolved in distilled water, and dialyzed against 0.01 M phosphate buffer, pH 6.5 for 24 hr. The dialysate was applied on a DEAE-Sephadex column (2 $\times$ 35 cm) previously equilibrated with 0.01 M phosphate buffer, pH 6.5. After washing with the same buffer, the column was eluted with 0.05 M phosphate buffer, pH 6.5, and active fractions (120 ml) were collected and lyophilized.

Step 6. SE-Sephadex column chromatography (first). The powder obtained in step 5 was dissolved in 0.1 M acetate buffer, pH 4.0 and the solution was dialyzed against the same buffer for 20 hr. The dialysate was applied on a SE-Sephadex column (1 $\times$ 26 cm) previously equilibrated with 0.1 M acetate buffer, pH 4.0, and the column was eluted with a linear gradient of concentration of NaCl in the same buffer. Active fractions (80 ml) were collected and dialyzed against 0.1 M acetate buffer, pH 4.0.

Step 7. SE-Sephadex column chromatography (second). The dialysate was again applied on

a SE-Sephadex column (1×20 cm) previously equilibrated with 0.1 M acetate buffer, pH 4.0. Elution was performed under the above mentioned condition (Fig. 1). Fractions No. 37 to 40 (40 ml) were collected, dialyzed against distilled water and lyophilized.

Step 8. Gel filtration with Sephadex G-100. The powder was dissolved in 2 ml of 0.1 M acetate buffer, pH 5.5, and the solution was applied on a Sephadex G-100 column (2×140 cm) previously equilibrated with the same

buffer. The column was eluted with the same buffer. The result is shown in Fig. 2.

Final preparation showed 940-fold purification over the crude extract. It was almost homogeneous on disc electrophoresis (Fig. 3), and was free from polygalacturonase, hemicellulase and protease. The purification of the enzyme is summarized in Table I.

Properties of the enzyme

Molecular weight. The molecular weight of

FIG. 1. SE-Sephadex Column Chromatogram.

SE-Sephadex column, 1×20 cm; buffer, 0.1 M acetate buffer, pH 4.0. NaCl gradient is shown in the figure. Ten ml fractions were collected.

— O.D._{280nm} ●—● PTE ---- NaCl

FIG. 2. Gel Filtration of PTE on Sephadex G-100.

Sephadex G-100 column, 2×140 cm; buffer, 0.1 M acetate buffer, pH 5.5. Five ml fractions were collected.

— O.D._{280nm} ●—● PTE

TABLE I. PURIFICATION OF PTE FROM *Aspergillus sojae* No. 48

Purification step	Volume (ml)	Total		Specific activity	Yield (%)
		PTE (units)	Protein (mg)		
1. Extraction	34,000	26,690	327,760	0.081	100
2. Ammonium sulfate fractionation	1,353	19,930	74,415	0.268	74.7
3. Batchwise treatment with DEAE-Sephadex	480	12,720	17,808	0.714	47.6
4. CM-Cellulose column chromatography	200	7,216	1,278	5.65	27.0
5. DEAE-Sephadex column chromatography	120	3,900	148.8	26.2	14.6
6. SE-Sephadex column chromatography (first)	80	1,900	34.0	55.9	7.1
7. SE-Sephadex column chromatography (second)	40	1029.6	14.2	72.5	3.9
8. Gel filtration with Sephadex G-100	35	397.8	5.2	76.5	1.5

FIG. 3. Disc Electrophoretic Pattern of Purified PTE.

FIG. 4. Estimation of Molecular Weight by Gel Filtration.

PTE and marker proteins (2 ml) were applied on a Sephadex column (2.5×115 cm) and eluted with 0.1 M acetate buffer, pH 5.0.

PTE was estimated as approximately 32,000 by the Andrews' gel filtration method¹⁴⁾ using myoglobin, chymotrypsinogen, pepsin and

FIG. 5. Effect of pH on PTE Activity.

Activity was measured in a reaction mixture containing 0.5 ml of enzyme, 1.0 ml of 0.1 M buffer and 1.0 ml of 1% pectin (or Link pectin) with or without calcium ion.

Acetate buffer was used for pH 4 to 6; Tris-acetate buffer for pH 7 to 9.

●—● Pectin
○—○ Pectin, with 5×10^{-2} M Ca ion
△—△ Link pectin.

ovalbumin as reference proteins (Fig. 4).

Effect of pH on the enzyme activity. The effect of pH on the activity of PTE is shown in Fig. 5. The pH optimum for the activity lies at 5.5 with pectin as substrate. But the addition of calcium ion (final concn. 5×10^{-2} M) to the reaction mixture, shifted pH optimum to 7.0 accompanied with the stimulation of the activity. If highly esterified pectin (Link pectin) was used as substrate, pH optimum was found to be around 7.0. When Link pectin was used as substrate, the addition of calcium ion did not cause the stimulation of activity and the change of pH optimum.

The effect of calcium ion was also observed in the reducing rate of viscosity of pectin. As shown in Fig. 6, the addition of calcium ion stimulated the reducing rate of viscosity

FIG. 6. Reduction of Viscosity of Pectin with or without Ca ion.

Reaction mixture containing 1.0 ml of enzyme, 3.0 ml of 1% pectin and 3.0 ml of 0.1 M acetate buffer, pH 5.5 (tris-acetate buffer for pH 7.0) with or without calcium ion, was incubated at 40°C in an Ostwald viscosimeter.

- without Ca ion, pH 5.5
- without Ca ion, pH 7.0
- with 5×10^{-2} M Ca ion, pH 5.5
- with 5×10^{-2} M Ca ion, pH 7.0

FIG. 7. Effect of pH on PTE Stability.

0.05 M sodium acetate-HCl buffer was used for pH 1.5 to 3.2; sodium acetate-acetate buffer for pH 4 to 6; tris-acetate buffer for pH 7 to 9.

- at 4°C for 24 hr
- at 30°C for 24 hr

at both pHs 5.5 and 7.0. And it was apparent that the reducing rate at pH 7.0 became higher than that at pH 5.5 by the addition

FIG. 8. Effect of Temperature on PTE Stability.

Enzyme was heated in 0.02 M acetate buffer, pH 5.5, at various temperatures for 10 min.

of calcium ion.

Stability. Effect of pH and temperature on the stability of PTE are shown in Figs. 7 and 8, respectively. The enzyme was quite stable between pH 4 and 7 when incubated at 4°C and 30°C. The enzyme was fairly acid-stable; after storage at pH 1.5 and 30°C for 24 hr, 54% of activity remained.

The enzyme was stable below 40°C at pH 5.5, but a rapid loss of activity was observed at higher temperatures. Treatment at 70°C for 10 min brought complete loss of activity.

Effect of metal ions on the enzyme activity. The effects of metal ions on the activity were tested using pectin and Link pectin as substrate in 0.05 M acetate buffer, pH 5.5. The addition of divalent cations such as Ni^{2+} , Cu^{2+} and Pb^{2+} to the reaction mixture containing pectin formed too much gel to measure enzyme activity. As shown in Table II, using pectin as substrate, most of divalent cations stimulated the activity by 15~25%. But when Link pectin was used, these metal ions had no effect on the activation of the enzyme. Both Cu^{2+} and Ag^+ had slight inhibitory effect on the activity and Hg^{2+} showed a remarkable inhibition.

As shown in Table III, optimum concen-

TABLE II. EFFECT OF METAL ION ON PTE ACTIVITY

Reaction mixtures contained 0.5 ml of enzyme, 2 ml of 0.5% pectin (or Link pectin) in 0.05 M acetate buffer, pH 5.5, and metal ion (final concentration, 10^{-3} M).

Metal ion	Activity (%)	
	Substrate	
	Pectin	Link pectin
No addition	100	100
K ⁺	108	103
Na ⁺	104	103
Mg ²⁺	116	98
Ca ²⁺	117	103
Mn ²⁺	121	103
Co ²⁺	123	103
Ni ²⁺	— ^{a)}	99
Cu ²⁺	— ^{a)}	81
Zn ²⁺	119	94
Ag ⁺	72	90
Cd ²⁺	113	111
Ba ²⁺	124	104
Hg ²⁺	19	25
Pb ²⁺	— ^{a)}	98

^{a)} formed gel by the addition of metal ion

TABLE III. OPTIMUM CONCENTRATION OF METAL ION

Reaction mixtures contained 0.5 ml of enzyme, 2 ml of 0.5% pectin in 0.05 M acetate buffer, pH 5.5, and metal ion.

Final conc. (M)	Activity (%)		
	Na ⁺	Mg ²⁺	Ca ²⁺
0	100	100	100
10^{-4} M	104	106	105
10^{-3} M	104	115	118
10^{-2} M	113	141	149
5×10^{-2} M	129	164	167
10^{-1} M	139	160	160
2×10^{-1} M	148	145	134
3×10^{-1} M	148	128	115

tration of metal ion for the activity using pectin as substrate was 5×10^{-2} M for divalent cation and $2 \sim 3 \times 10^{-1}$ M for monovalent cation.

TABLE IV. EFFECT OF VARIOUS SUBSTANCES ON PTE ACTIVITY

Enzyme and test substances were pre-incubated in 0.05 M acetate buffer, pH 5.5, for 10 min at 30°C.

Substance	Concn.	Relative activity (%)
No addition	10^{-3} M	100
EDTA	10^{-3} M	96
<i>o</i> -Phenanthroline	10^{-3} M	99
8-Hydroxyquinoline	10^{-3} M	86
<i>p</i> -Chloromercuribenzoate	2×10^{-4} M	90
Monoiodoacetic acid	10^{-3} M	98
Sodium thioglycolate	10^{-3} M	92
Cysteine	10^{-3} M	98
Potassium cyanide	10^{-3} M	100
N-Bromosuccinimide	10^{-3} M	2
Iodine	10^{-3} M	11

TABLE V. ELIMINATIVE CLEAVAGE OF POLYURONIC COMPOUNDS BY PURIFIED ENZYME

Reaction mixtures containing 0.5 ml of enzyme, 1.0 ml of 1% substrate and 1.0 ml of McIlvaine buffer, were incubated at 40°C for 60 min.

Substrate	pH	Increase in absorbance at 235 m μ
Pectin	5.5	31.2
Link pectin	5.5	60.8
Pectic acid	4.5	0
Alginic acid	5.5	0
Chondroitinsulfate	5.0	0

Effect of various substances on the enzyme activity. As shown in Table IV, metal chelating reagents did not show inhibitory effect on the activity. The enzyme activity was not affected by reducing reagents and those reagents which convert SH groups in protein into mercaptides. In contrast, such oxidizing reagents as N-bromosuccinimide and iodine caused severe inactivation.

Specificity. The purified enzyme was specific for α -1, 4 methylesterified galacturonide linkage. It could not attack α -1, 4 nonesterified galacturonide linkage (pectic acid), β -1, 4

FIG. 9. A) Absorption Spectrum of Reaction Products. B) Absorption Spectrum of Thiobarbituric Acid Reaction Products.

FIG. 10. Cleavage of Glycosidic Bond by PTE

Reaction mixture contained 200 mg of substrate in 20 ml of 0.05 M acetate buffer, pH 5.5 (0.05 M tris-acetate buffer, pH 7.0 for Link pectin) and 17 units of enzyme. Temperature: 40°C.

- Pectin
- Pectin, with 5×10^{-2} M Ca ion
- △—△ Link pectin

mannuronide and guluronide linkage (alginic acid), and chondroitin sulfate (Table V).

Absorption spectrum of reaction products. The absorption spectrum of reaction products of Link pectin in the ultraviolet region is shown in Fig. 9 A. The reaction products, supposedly 4, 5-unsaturated methyl galacturonides, had an absorption maximum at 238 mμ. This value was rather higher than that reported

by Albersheim *et al.*¹¹

When 4, 5-unsaturated methyl galacturonides were allowed to react with thiobarbituric acid,¹⁶ the reaction products had an absorption maximum around 552 mμ (Fig. 9 B). This value was also higher than that reported by Albersheim *et al.*¹¹

Cleavage of glycosidic linkage by PTE. The rates of cleavage of glycosidic linkages in pectin and Link pectin were followed by measuring the liberated aldehyde groups determined by iodometric method.¹⁶ When pectin was used as substrate, the experiments were done both in the presence and absence of 5×10^{-2} M CaCl_2 in the reaction mixture. The results are shown in Fig. 10. Calcium ion increased the initial rate of reaction, but had no effect on the degradation extent of pectin, which was 26.3% after 48 hr. On the other hand, the degradation of Link pectin extended up to 39.7% after 72 hr.

DISCUSSION

PTE from *Asp. sojae* was purified by means of ammonium sulfate fractionation, batchwise treatment with DEAE-Sephadex, ion exchange column chromatography and gel filtration. The purified enzyme was almost homogeneous on disc electrophoresis and was free from polygalacturonase, hemicellulase, cellulase and protease. But the yield of final preparation was very low.

Molecular weight of PTE, which had not been reported previously, was about 32,000 in *Asp. sojae*. This value is quite similar to those of endo-pectic acid transeliminase from *Erwinia carotovora* (31,000)¹⁷ and *Erwinia chrysanthemi* (30,000~32,400).¹⁸

Optimum pH of PTE was 5.5 with pectin as substrate. This pH value is close to that reported previously.^{1,7} But when Link pectin was used as substrate, pH optimum of the enzyme exhibited 7.0. And if 5×10^{-2} M CaCl_2 was added to the reaction mixture, the pH

optimum for pectin shifted to neutral side. The same effect of calcium ion for pectin was observed in the reducing rate of viscosity.

Only when pectin was used as substrate, activity of PTE was stimulated by divalent cations. As shown in Table III, optimum concentration of divalent cation was found to be high. Although calcium ion increased the initial rate of reaction, it did not extend the limit of degradation of pectin.

These results suggested that cations, especially divalent cations might have a strong affinity for about 30% of free carboxyl groups in pectin, and reduce the negative charge of the substrate especially at pH above 5.5. Consequently, pectin will have more affinity for the enzyme. This resulted in the stimulation of activity and the shift of pH optimum by the addition of divalent cations.

PTE of *Asp. sojae* degraded glycosidic linkages in pectin and Link pectin up to 26.3 and 39.7%, respectively. These degradation extents were higher than those obtained by *Asp. fonsecaeus*.⁷⁾ The result suggests that the specificity of *Asp. sojae* PTE for methyl esterified oligogalacturonides may be rather lower than that of *Asp. fonsecaeus* PTE. Since PTE from *Asp. fonsecaeus* could not attack the trimer and low methyl esterified galacturonides, exhaustive degradation of Link pectin by the enzyme yielded mainly unsaturated tri-, tetra- and penta-methyl esterified galacturonides.⁷⁾ But in the case of *Asp. sojae*, low methyl esterified galacturonides than trimer seem to have been formed from Link pectin as detected by paper chromatography and gel filtration. Further studies will be done to identify these reaction products.

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