Some Properties of Endo-polygalacturonase from *Trichosporon penicillatum* SNO-3[†]

Takuo SAKAI,* Minoru OKUSHIMA and Masahiko SAWADA

Department of Agricultural Chemistry, College of Agriculture,
University of Osaka Prefecture, Sakai, Osaka 591, Japan

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Some properties of the endo-polygalacturonase from *Trichosporon penicillatum* were investigated. The enzyme showed the highest activity around pH 5.0 and was stable at this pH up to 50°C. The enzyme catalyzed the hydrolysis of galacturonic acid oligomers as well as its polymer. The pentamer was degraded to a trimer and a dimer, the tetramer to a trimer and a monomer, and the trimer to a dimer and a monomer, respectively, whereas the dimer was not degraded. The kinetic constant $V_{\max}$ and K_m values changed with the substrate chain-length; the K_m values tended to decrease, whereas the $V_{\max}$ values tended to increase with increasing chain-length of the substrate. The amino acid residue participating in the active site of the enzyme was studied and it was found to be histidine.

There have been many observations on the maceration ability of endo-polygalacturonase and this activity is applied in the production of fruit juice and wines.

Some strains of yeast producing endo-polygalacturonase have been found and these enzymes have the ability to clarify a citrus pectin medium. However, the papers on endo-polygalacturonases published so far have not dealt with their protopectin-solubilizing activity. In the preceding papers, the authors presented an endo-polygalacturonase from *Trichosporon penicillatum* having protopectin-solubilizing activity, and demonstrated a new method for pectin production using the enzyme.^{1~4)}

The present paper describes some properties of this endo-polygalacturonase having protopectin-solubilizing activity produced in a culture filtrate of *Trichosporon penicillatum* SNO-3.

MATERIALS AND METHODS

Chemicals. All chemicals, unless otherwise specified, were of Wako Pure Chemicals Industries (Osaka) certified

reagent grade. Protopectin was prepared by the method described in the preceding paper.⁴⁾

Enzyme. The enzyme used in this study was a crystalline preparation which was obtained by the method described in the preceding paper.⁴⁾

Preparation of oligogalacturonic acids. Oligogalacturonic acids were prepared according to the method described by Nagel *et al.*⁵⁾ and isolated by chromatography on a Dowex 1 × 8 (200~400 mesh) column. Their purities were confirmed by thin layer chromatography (TLC), and their polymerization degrees were determined from the ratio of total reducing groups to total galacturonic acid molecules. Thin layer chromatography was performed using a cellulose sheet (Kodak Chromato Sheet®, the solvent system used was the epiphase from a mixture of ethylacetate-acetic acid-water=2:1:2). The individual galacturonic acid derivatives on TLC were revealed with a periodate-benzidine reagent.⁶⁾

Preparation of polygalacturonic acids with various numbers of methoxyl groups. Polygalacturonic acids with various numbers of methoxyl groups were prepared as follows: To 100 ml of 1% pectin solution was added 100 ml of 0.1 N NaOH solution and this mixture was stood at 0°C for up to 45 min (this period depended upon the methoxyl group content of the product). After the reaction, 1 ml of 16 N acetic acid solution and 600 ml of ethanol were added in that order. The resultant precipitate was collected by centrifugation (2,000 × *g* for 20 min) at 5°C, washed with

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* To whom correspondence should be addressed.

acetone, dissolved in distilled water, and dialyzed against distilled water overnight. The dialyzed solution was lyophilized and used as the sample.

Enzyme assay. Except for the determination of K_m and V_{max} , enzyme activities were determined by the method described in the preceding paper.⁴⁾ To determine K_m and V_{max} values, the enzyme reaction was performed at 37°C, and the activity was quantified by measuring the release of reducing groups from oligogalacturonic acid using the Somogyi assay method⁷⁾ with Nelson's arsenomolybdate reagent.⁸⁾

Photoinactivation of the enzyme. The photoinactivation experiments were performed at 20°C with the enzyme solution (20 µg/ml) containing 0.001% Methylene Blue and 0.05 M Tris-HCl, pH 7.5, in a 15 × 150 mm tube (sample volume of 2 ml). The test tube containing the enzyme solution was placed 10 cm from a 150 W projector.

Analyses. The protein concentration was determined by the method of Lowry *et al.*⁹⁾ using bovine serum albumin as a standard.

Amino acid analysis was performed on a Hitachi KLA-5 Amino Acid Analyzer.

RESULTS

Effect of pH on activity and stability of the enzyme

The effect of pH on protopectinase and pectic acid liquefying activities was investigated and the results are shown in Fig. 1. The pH-activity profiles changed depending upon the substrate used, and showed maximum activity around pH 5.5 for protopectinase and around 5.0 for pectic acid liquefying activity, respectively.

The effect of pH on the stability of the enzyme was investigated by preincubation for 30 min at 37°C in various buffers of pH 2 to 11. The enzyme activity was not lost in the acidic pH range of 3 to 6, but the activity was lost remarkably at alkaline pHs (higher than pH 7.5) (Fig. 2).

Effect of temperature on activity and stability of the enzyme

The optimal temperature for enzyme activity changed depending upon the substrate used, being around 50°C for pectic acid and 40°C for protopectin (Fig. 3). The enzyme was

FIG. 1. Effect of pH on the Enzyme Activity.

The pectic acid liquefying activity was assayed in 0.02 M MacIlvaine buffer and protopectinase activity was assayed in 0.02 M acetate buffer (pH 4 to 5.5) and 0.02 M sodium phosphate buffer (pH 5.5 to 7.0), respectively, under the standard assay conditions. (●), pectic acid liquefying activity; (○), protopectinase activity.

FIG. 2. Effect of pH on the Enzyme Stability.

The enzyme (30 units/ml) in various buffers (0.02 M acetate buffer, pH 3 to 5.5, and 0.02 M sodium phosphate buffer, pH 6.0 to 9.0) containing 50 µg/ml bovine serum albumin was incubated at 37°C for 30 min, and remaining activities were determined under the standard assay conditions. (●), pectic acid liquefying activity; (○), protopectinase activity.

treated for 30 min (at pH 5.0) at the temperatures indicated in Fig. 4, and then the remaining activity was assayed. The enzyme was

FIG. 3. Effect of Temperature on the Enzyme Activity. The enzyme activities were assayed at various temperatures (30 to 60°C) under the standard assay conditions. (●), pectic acid liquefying activity; (○), protopectinase activity.

FIG. 4. Effect of Temperature on Stability of the Enzyme.

The enzyme (30 units/ml) in 0.02 M acetate buffer, pH 5.0, containing 50 μ g/ml bovine serum albumin was kept for 30 min at various temperatures (30 to 60°C), and the remaining activities were assayed under the standard assay conditions. (●), pectic acid liquefying activity; (○), protopectinase activity.

stable at temperatures lower than 50°C, but unstable above 55°C, and lost its activity remarkably at 60°C under the conditions used in this experiment.

Effects of various compounds on enzyme activity

The effects of some compounds on enzyme

TABLE I. EFFECT OF VARIOUS COMPOUNDS ON ENZYME ACTIVITY

Enzyme activity was determined as pectic acid liquefying activity.

Compound	Concentration (mM)	Relative activity
None	—	100
AgNO ₃	0.1	87
	1.0	44
Ba(CH ₃ COO) ₂	1.0	100
CaCl ₂	1.0	44
CoCl ₂	1.0	44
CuSO ₄	1.0	62
EDTA ^a	0.1	100
FeCl ₂	1.0	92
KCl	1.0	108
KCN	0.1	100
LiSO ₄	1.0	100
MgSO ₄	1.0	100
MnCl ₂	1.0	92
NaCl	1.0	100
NaN ₃	0.1	100
Pb(CH ₃ COO) ₂	0.1	108
SnCl ₂	0.1	100
ZnCl ₂	0.1	100
Iodoacetic acid	0.1	87
	1.0	84
Mersalyl acid	0.05	70
	0.1	45
<i>p</i> -Chloromercuribenzoic acid	0.01	100
	0.05	73
	0.1	15
<i>p</i> -Chloromercuriphenylsulfonic acid	0.1	93

^a Ethylenediaminetetraacetic acid.

activity (pectic acid liquefying activity) were determined at pH 5.0. As shown in Table I, the enzyme activity was inhibited by such metal ions as Ag²⁺, Ca²⁺, Co²⁺, Cu²⁺, Hg²⁺ and Hg⁺.

Action of the enzyme on oligogalacturonic acids

The enzyme activity on the hydrolysis of galacturonic acid oligomers was determined. Figure 5 shows thin layer chromatograms of products of the enzyme reaction with tetra- and pentagalacturonic acid. Trigalacturonic acid and digalacturonic acid were the reaction products of pentagalacturonic acid, and galacturonic acid and its dimer were the reaction

FIG. 5. Thin Layer Chromatograms of the Time Course of Hydrolysis of Tetra- and Pentagalacturonic Acid by the Enzyme.

Reaction mixtures contained 20 mg of the respective oligogalacturonic acid in 0.4 ml of 0.02 M acetate buffer, pH 5.0, and 0.1 ml of the enzyme solution (containing 2 μ g of enzyme), and reactions were performed at 37°C for the indicated periods (1, 0; 2, 2; 3, 6; 4, 15; 5, 30; and 6, 100 min, respectively). After incubation, 50 μ l of a reaction mixture was mixed with 0.05 μ l of 1 M mercurous acetate solution to stop the reaction and a 2 μ l aliquot was subjected to thin layer chromatography.

A, chromatogram of the reaction products with the enzyme from tetragalacturonic acid; B, chromatogram of the reaction products with the enzyme from pentagalacturonic acid.

a, b, c, d, and e indicate the positions of galacturonic acid, its dimer, trimer, tetramer and pentamer, respectively.

TABLE II. KINETIC PROPERTIES OF THE ENZYME

The reaction mixture was composed of 0 to 200 μ mol substrate, 10 mmol acetate buffer, pH 5.0, and enzyme solution (0.5 units for polygalacturonic acid, 5 units for pentagalacturonic acid, 20 units for tetra- and trigalacturonic acid and 50 units for digalacturonic acid, respectively) in a total volume of 2.0 ml, and the reaction was carried out at 37°C for 5 and 10 min for polygalacturonic acid, 5 and 10 min for pentagalacturonic acid, 60 and 90 min for tetragalacturonic acid and 90 and 180 min for trigalacturonic acid, respectively.

Substrate	Michaelis constant (mM)	Maximal velocity (μ mol/mg protein/min)
Digalacturonic acid	Not reacted in 24 hr	
Trigalacturonic acid	4.26	2.76
Tetragalacturonic acid	2.20	300
Pentagalacturonic acid	0.87	1644
Polygalacturonic acid (containing 270 galacturonic acid residues)	0.04	13386

products of tetragalacturonic acid. Galacturonic acid and its dimer were formed by the reaction with trigalacturonic acid. However, degradation of digalacturonic acid by the enzyme was not observed.

As shown in Table II, listing the K_m and V_{max} values, the rate of hydrolysis catalyzed by the enzyme decreased with shortening of the oligogalacturonic acid chain-length. Trigalacturonic acid was the lowest oligogalac-

turonic acid which was hydrolyzed by the enzyme, and it was hydrolyzed extremely slowly; Its $V_{\max}$ was approximately two orders lower than that of tetragalacturonic acid.

Relationship between the methoxyl group content of the substrate and the molecular weight of its degradation products with the enzyme

The effect of the methoxyl group content of the substrate on the polymerization degree of the reaction product was investigated using polygalacturonic acid with methoxyl groups of various numbers as the substrate, and the results are shown in Fig. 6. The plot of content of galacturonic acid with methoxyl groups (in percent) of the substrate was parallel to the logarithm of the polymerization degree of the reaction products.

Survey of amino acid residues participating in the enzyme reaction

In order to survey the amino acids participating in our enzyme reaction, some studies were performed.

The enzyme was inactivated by illumination

of the enzyme solution in the presence of Methylene Blue, and loss of activity was observed to follow first order kinetics (Fig. 7), but not with either both Methylene Blue or illumination alone. The rates of photoinactivation in the presence of Methylene Blue were pH-dependent and remaining activity *versus* pH is shown in Fig. 8, and the pK value was calculated to be around pH 6.1. The pH-dependence of the photoinactivation indicated that an amino acid residue which dissociated around pH 6.1 was essential for the enzyme's active site. This was an indication that a histidyl residue might be being photo-oxidized, since the dye-sensitized photo-oxidation of histidine has been shown to be sharply pH-dependent, the protonated form being resistant to oxidation.¹⁰⁾ In order to confirm this supposition, investigations were done on the relationship between enzyme activity lost and decomposed amino acids which are known to be sensitive to photo-oxidation. The photo-oxidized enzyme solution was dialyzed thoroughly against distilled water, hydrolyzed in 6 N HCl containing 3% thioglycolic acid in

FIG. 6. Relationship between Methoxyl Groups of the Substrate and the Molecular Weight of Its Degradation Product with the Enzyme Reaction.

Reactions were performed under the standard assay conditions using polygalacturonic acid (mean polymerization degree, 33) containing the various numbers of methoxyl groups indicated. Polymerization degrees were determined from the ratio of total reducing groups to total galacturonic acid molecules.

FIG. 7. Effect of pH of the Rates of Photoinactivation of the Enzyme in the Presence of Methylene Blue. Illumination of the enzyme solution was performed under the conditions given in MATERIALS AND METHODS. Activity loss of the enzyme at pH 5.0 (■), pH 5.5 (□), pH 6.0 (●) and pH 6.5 (▲), in the absence of Methylene Blue (△) and when not illuminated (○).

FIG. 8. The pH-Dependence of the Photoinactivation of the Enzyme.

Illumination of the enzyme solution was performed under the conditions given in MATERIALS AND METHODS, using 0.1 M citrate-NaOH buffer (●), and 0.1 M potassium phosphate buffer (○). In the figure, an arrow indicates the pK value of photoinactivation of the enzyme.

a sealed tube filled with nitrogen gas at 100°C for 36 hr, and applied to the amino acid analyzer. The results are shown in Fig. 9. Tryptophan was found to be decomposed by photo-oxidation as well as histidine, whereas tyrosine (known as an amino acid decomposed by photo-oxidation) was not decomposed. Enzyme activity was lost parallel with the amount of histidine decomposed, but not with that of tryptophan (even though tryptophan

FIG. 9. Relationship between Decrease of Amino Acid and the Enzyme Activity on Photooxidation.

Illumination of the enzyme was performed under the conditions given in MATERIALS AND METHODS up to 90 min, and aliquots of the illuminated enzyme solution were dialyzed against distilled water, hydrolyzed in 6N HCl containing 3% thioglycolic acid in a sealed tube filled with nitrogen gas at 100°C for 36 hr and applied to an amino acid analyzer. (▲), tyrosine; (●), tryptophan; (○), histidine.

was not decomposed, about 70% of the initial enzyme activity was lost).

Effect of galacturonic acid or its oligomers on the enzyme activity

The effects of galacturonic acid or its oligomers on protopectinase and pectic acid

FIG. 10. Effect of Galacturonic Acid or Its Oligomers on the Enzyme Activity.

The reaction was performed under the standard assay conditions except that galacturonic acid (●), digalacturonic acid (○) or trigalacturonic acid (▲) was added as indicated.

A, effect on protopectinase activity; B, effect on pectic acid liquefying activity.

liquefying activity were determined. As shown in Fig. 10, pectic acid liquefying activity was inhibited by galacturonic acid and its dimer and trimer, whereas protopectinase activity was not inhibited.

DISCUSSION

As our enzyme is intended to be used for industrial purposes, the effects of pH and temperature on activity and stability of the enzyme were investigated, first. Although the optimal pH of the enzyme reaction varies depending upon the substrate used (pectic acid and protopectin were used as substrates), they lie around pH 5.0. These properties are favor-

able for practical use, because the enzyme is to be used in pectin production and pectin is most stable around pH 5.0. The enzyme is stable at acidic pHs (pH 3 to 6) up to 50°C. However, the stability of pectic acid liquefying and that of protopectinase activity are different at higher than pH 7.0; pectic acid liquefying activity is stable up to pH 8.0, whereas protopectinase activity is lost higher than pH 7.0. We suppose that the enzyme has a site having affinity to protopectin and a site that is labile at alkaline pH. However, we did not study this problem further.

Most polygalacturonases have optimal pHs in the weakly acidic region between pH 4.0 and 6.5.^{11~14)} Endo-polygalacturonase from *Saccharomyces fragilis* shows a pH optimum with pectic acid at pH 4.4, whereas with oligogalacturonic acids the pH optimum is around pH 3.5.¹⁵⁾ A similar shift of pH optimum is observed with other endo-polygalacturonases.^{16~18)} With our enzyme, the pH optimum is different with pectic acid than with protopectin as a substrate. However, the mechanism of the shift of pH optimum was not studied.

The enzyme catalyzes the hydrolysis of galacturonic acid oligomers; pentagalacturonic acid is degraded to tri- and digalacturonic acid, tetragalacturonic acid to trigalacturonic acid and galacturonic acid, and trigalacturonic acid to digalacturonic acid and galacturonic acid, respectively, whereas digalacturonic acid is not degraded further.

Three different patterns of action towards galacturonic acid oligomers are known for endo-polygalacturonases.¹⁹⁾ This enzyme is novel in the criterion of action patterns towards oligogalacturonic acids.

The kinetic constant $V_{\max}$ and K_m values change with the substrate chain-length; the K_m values tend to decrease, whereas the $V_{\max}$ values tend to increase with increasing chain-length of the substrate. A great difference in $V_{\max}$ was found between trigalacturonic acid and tetragalacturonic acid. On the other hand, it was found that the methoxyl group content of the substrate affects the molecular weight of

the reaction products; the molecular weight of the reaction products increases in accordance with the increasing galacturonic acid with methoxyl groups of the substrate. As mentioned in the preceding paper, our enzyme reacts with protopectin and releases high molecular weight pectin (protopectinase activity). On the basis of these results, we assume the mechanism of protopectinase activity of the enzyme to be as follows: The enzyme reacts with the pectin molecule in protopectin at sites having more than three nonmethoxylated-galacturonic acid-chains (realistically more than four nonmethoxylated-galacturonic acid chains, considering the reaction velocities for galacturonic acid oligomers) and splits their glycoside bonds. Generally, 50% of the galacturonic acid of pectin in protopectin is methoxylated at random. Therefore, the pectin molecule in protopectin may be split at restricted sites, so as to form high molecular weight pectin. Moreover, as shown in Fig. 10, galacturonic acid, its dimer and trimer, which may be produced in the protopectinase reaction, all inhibit pectin degradation activity, whereas protopectinase activity is not inhibited, and the pectin molecules released from protopectin are protected from further degradation. Thus, our enzyme releases high molecular weight pectin from protopectin.

Many pectolytic enzymes, including endo-polygalacturonase, pectin lyase and pectate lyase, are known to show maceration activity on various plant tissues. Our enzyme showed maceration activity on potato tissue but not on citrus peel. This phenomenon seems to indicate the diversity of plant tissues. From this we assumed that protopectinase activity does not always accompany maceration activity. However, details of this problem remain to be clarified.

Some studies on the amino acids participating in the active sites of endo-polygalacturonases have been published. On the basis of the effect of pH on the activity of *Aspergillus niger* endo-polygalacturonase, Koller²⁰⁾ considered that essential groups for

the active site are the imidazole group of the histidine and the carboxyl groups of the glutamic acid or aspartic acid residues of the enzyme. After that, Rexová-Benková and Slezárik^{21,22)} showed that the inactivation of the enzyme was paralleled by the decomposition of histidine and indicated that one of the five histidine residues present in the enzyme molecule was essential for its activity.

In the present study, similar results to those of Rexová-Benková and Slezárik were obtained. Namely, the enzyme is inactivated by photo-oxidation in the presence of Methylene Blue, and the inactivation of the enzyme is paralleled by the decomposition of histidine in the enzyme molecule. These facts indicate that a histidine residue is essential for the activity of our enzyme.

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