Protopectin-solubilizing Enzyme from *Trichosporon penicillatum*

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Protopectin is the water-insoluble parent pectic substance which occurs in plants. Upon limited hydrolysis, it yields pectins of various molecular weights. Protopectinase supposedly hydrolyzes protopectin into pectin, and has been assumed to be different from common pectolytic enzyme.¹⁾ Recently, many successful studies on the pectolytic enzyme have been performed, and some enzymes that act on middle lamella pectin (macerating enzyme) have been isolated from microbial sources.^{2~6)} From the results, protopectinase has been considered to be macerating enzyme, but not to be a distinct enzyme from pectolytic enzyme. However, there are few studies on the enzyme that catalyzes the release of pectin of large molecular weight from protopectin.

The authors isolated a yeast which produces an enzyme that catalyzes the release of pectin of large molecular weight from citrus (*Citrus unshiu*) peel without macerating the tissues. This strain was identified as a variety of *Trichosporon penicillatum* and was named strain SNO-3. This paper deals with the purification of the enzyme having protopectinase activity (tentatively called protopectinase in this paper) produced by strain SNO-3.

Strain SNO-3 was aerobically grown on a medium containing 2% glucose, 0.4% polypepton, 0.2% yeast extract, and 0.08% silicone KM-70, pH 5.0. After a 25-hr incubation at 30°C, the cultured broth was centrifuged to remove the yeast cells, the resultant supernatant was concentrated to one-tenth of its original volume, and then used as the starting material for enzyme purification. Protopectinase activity was determined by measuring

the amount of pectic substance released from protopectin (prepared from citrus peel) by the carbazole-sulfuric acid method.⁷⁾ The enzyme was purified as follows: The starting material was fractionated by ammonium sulfate, and the fraction precipitated from between 40 and 80% saturation of the ammonium sulfate was collected, dissolved in 0.02 M acetate buffer, pH 5.0, and dialyzed against the same buffer. Dialyzed enzyme solution was applied to a CM-Sephadex C-50 column equilibrated with 0.02 M acetate buffer, pH 5.0, and eluted by a linear gradient of NaCl (0 to 0.4 M). Finally, the enzyme was purified by chromatography on a Sephadex G-75 column equilibrated with 0.02 M acetate buffer, pH 5.0. The overall yield of the enzyme activity was about 75% that of the starting material, and 73 mg of enzyme was obtained from 40 liters

FIG. 1. Microphotograph of Crystals of Protopectinase from *Trichosporon penicillatum* SNO-3.

FIG. 2. Polyacrylamide Gel Electrophoresis in the Absence (A) and Presence (B) of Sodium Dodecyl Sulfate.

(A): Electrophoresis was performed on the pH 4.0 system of Davis.⁸⁾ Electrophoresis was carried out from anode to cathode.

(B): Electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed by the method of Weber and Osborn.⁹⁾ Electrophoresis was carried out from cathode to anode.

of cultured broth. The enzyme was then crystallized in the presence of ammonium sulfate. Figure 1 shows crystals of the enzyme. The crystalline enzyme showed a single band on polyacrylamide gel electrophoresis at pH 4.0 (Fig. 2), and its homogeneity was also confirmed by ultracentrifugal analysis. The molecular weight of the enzyme was determined to be 27,000 by means of gel-filtration on a Sephadex G-75 column, and its isoelectric point was determined to be pH 7.8~8.0 by a isoelectrofocusing method.¹⁰⁾ The enzyme acted on protopectin yielding pectic substance, which was analyzed by gel-filtration on a Sepharose 6B column, and which was observed to have a molecular weight larger than 200,000 (Fig. 3). Because of this, the present

enzyme was considered to be a kind of protopectinase. The present enzyme catalyzed the hydrolysis of pectic acid and remarkably decreased the viscosity of the pectic acid-containing reaction medium. The enzyme increased its reducing value very slightly (Fig. 4). The enzyme did not catalyze transeminative cleavage of pectic acid. From these observations, the authors considered this enzyme to be an endo-polygalacturonase.

A microbial enzyme that catalyzes solubilization of protopectin was first demonstrated by Kaji,^{2,3)} who obtained a macerating enzyme as a single protein preparation from cultured broth of *Clostridium felsineum* var. *sikokianum*. He found that the enzyme converted the middle lamella pectin in the barks

FIG. 3. Gel-filtration Pattern of Pectin Released from Protopectin by the Action of Protopectinase.

One hundred milligrams of protopectin prepared from mandarin peel was suspended in 5 ml of 0.02 M acetate buffer, pH 5.0, and incubated with 60 μ g of purified protopectinase at 37°C for 60 min. After reaction, the mixture was filtered and 1 ml of the filtrate was used for analysis. The sample was applied to a 2 $\times$ 38 cm Sepharose 6B column. The column was eluted with 0.2 M NaCl at a flow rate of 7 ml/hr, and 4-ml fractions were collected. Pectin and dextran were determined by the carbazole-sulfuric acid method and the phenol-sulfuric acid method,¹¹⁾ respectively. The markers A and B represent, respectively, the elution volumes of blue dextran (mol. wt. 2,000,000) and cytosine (mol. wt. 111.1). ○—○, pectin (reaction product); ●—●, pectin (commercial product); ▲—▲, dextran (mol. wt. 200,000).

FIG. 4. Action of Protopectinase on Pectic Acid.

Liquefying activity on pectic acid was determined by the decrease of viscosity in a 0.43% pectic acid solution in 0.02 M McIlvaine buffer, pH 5.0, at 37°C (0.06 μ g/ml of enzyme was used). The rate of viscosity decrease (A) was calculated by the equation

$$A = \frac{T_a - T}{T_a - T_o} \times 100.$$

T_a : flow time (in seconds) of pectic acid solution added to the inactivated enzyme.

T : flow time (in seconds) of reaction medium, kept for the indicated period.

T_o : flow time (in seconds) of water added to the inactivation enzyme.

Reducing activity on pectic acid was determined as follows: A reaction mixture containing 0.43% pectic acid in 0.02 M McIlvaine buffer, pH 5.0, and 0.06 μ g/ml of enzyme was incubated at 37°C for the indicated period. The reducing activity was determined by the method of Jansen and MacDonnell.¹²⁾ ○—○, liquefying activity; ●—●, reducing activity.

of Gampi (*Wikstroemia sikokiana* Fr. et Sav.) to a soluble pectic substance. Enzymes having activity to release pectin from plant tissues were also demonstrated by Ishii⁶⁾: He showed that endo-pectin lyase and endo-polygalacturonase from *Aspergillus japonicus* catalyzed the release of pectin (though not pectin having a large molecular weight) from vegetable tissues.

The results obtained in this paper indicate that the present enzyme degrades protopectin to pectin of the larger molecular weight than that produced by any protopectin-solubilizing enzyme reported. It possibly depend on the unique specificity of the enzyme on pectic substance (Fig. 4).

From the facts, the present enzyme is considered to be different from any protopectin-solubilizing enzyme reported so far.

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