Short Communication

Protopectin Solubilizing Enzyme That Does Not Catalyze the Degradation of Polygalacturonic Acid

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Protopectin is a water-insoluble parent substance of pectin found in plant tissues. The reasons for pectin in plant tissue being insoluble are complicated, and include (1) binding of pectin molecules with polyvalent ions (such as Ca^{2+}, Mg^{2+}, and Fe^{2+}), leading to insolubility, (2) secondary valency bonding between pectin molecules with other constituents of the cell wall such as cellulose or hemicellulose, (3) salt bridging between the carboxyl groups of pectin molecules and basic groups of protein, and (4) mechanical entwining of the pectin molecules with each other and with other cell-wall constituents.[1]

Protopectin is solubilized by its restricted hydrolysis. The enzyme that catalyzes the solubilization of protopectin, which liberates a highly polymerized water-soluble pectin, was originally named protopectinase.[2]

In 1978, protopectinase was found in a yeast, and other microbial protopectinases have been isolated.[3～6] All of these enzymes catalyze the restricted hydrolysis of the polygalacturonic acid domain in insoluble pectin molecules, liberating water-insoluble pectin. These enzymes have been classified as endo-polygalacturonase [EC 3.2.1.15, poly(1,4-α-D-galacturonide)glycanohydrolase]. However, because the insolubility of pectin in plant tissue involves more than one factor, we thought that other kinds of protopectinase might exist. We screened for such new kinds of protopectinase.

This paper deals with a new kind of protopectinase that does not degrade polygalacturonic acid.

The organisms used were supplied from the stock of the Institute for Fermentation Osaka (Japan). They were cultured in a medium containing 0.5% soluble starch, 0.05% yeast extract, 1% $(NH_4)_2PO_4$, 0.05% $CaCl_2 \cdot 2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.02% KCl, pH 6.8, in a 500-ml shaking flask containing 100 ml of medium, on a shaker (120 rpm) at 37°C for 48 hr. Protopectinase activity was assayed by measuring the amount of pectin released from protopectin (prepared from lemon peel) as described elsewhere.[4] Polygalacturonase activity was assayed by measuring the release of reducing groups by the method of Jansen and MacDonnell.[7] The enzymatically extracted pectin was isolated by a method described before.[8]

We found a new kind of protopectinase that did not catalyze the degradation of polygalacturonic acid in culture broths of some *Bacillus* species such as *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. macerans*, *B. pumilus*, and *B. subtilis*. *B. subtilis* IFO 12113 produced this protopectinase in its culture borth at higher concentrations than the other species. The enzyme was purified and isolated from the culture filtrate of *B. subtilis* IFO 12113 as follows. The culture filtrate (20 l) was concentrated to 2 l and dialyzed against 20 mM acetate buffer (pH 5.0) (AcB) by ultrafiltration with a Labomodule SEP 1013 hollow fiber ultrafilter (Asahi Chemical Industry Co., Osaka, Japan), and then used as the starting material for enzyme purification. The enzyme solution (about 2 l) was put on a column (5×46 cm) of CM-Sephadex C-50 equilibrated with AcB. After the column was washed thoroughly with AcB, the adsorbed enzyme was eluted by a linear gradient of

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NaCl (from 0 to 500 mM) in 2 l of AcB. The fractions containing enzyme activity were pooled and ethylenediaminetetraacetic acid was added to the concentration of 50 mM. The mixture (about 255 ml) was heated at 60°C for 7 min, cooled rapidly, dialyzed against AcB, and passed through a column (2.1 × 15 cm) of DEAE-Toyopearl 650 (Tosoh Corp., Tokyo) equilibrated with AcB. The effluents were collected and concentrated to 4 ml; finally, the enzyme was purified by chromatography on a column (2.5 × 75 cm) of Toyopearl HW-55S (Tosoh Corp.) equilibrated with AcB containing 200 mM KCl. By this

FIG. 1. Polyacrylamide Gel Electrophoreses of Protopectinase-B in the Presence (a) and the Absence (b) of SDS.

Electrophoreses were done as reported elsewhere.[9,10] (a) A 40-μg sample was put on a 7% gel column and run at pH 4.5 for 2.5 hr at 5 mA per column. (b) A 40-μg sample treated with SDS at 100°C for 5 min was put on a 10% gel containing 0.1% SDS and run at 7 mA per column for 5 hr. The direction of electrophoresis was from left to right.

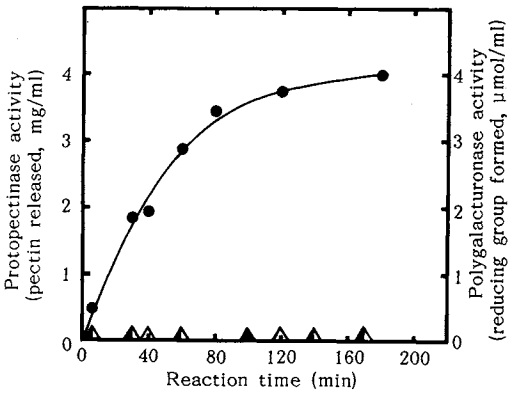

FIG. 2. Course of Enzyme Reaction of Protopectinase-B.

A reaction mixture containing 20 mg of protopectin or 15 mg of polygalacturonic acid or methoxylated polygalacturonic acid (with 75% of the carboxyl groups being methoxylated), 18 μg of protopectinase-B, and 100 μmol of acetate buffer (pH 5.0) per milliliter was incubated at 37°C for the indicated time. ●, protopectinase activity; △, polygalacturonase activity (substrate was polygalacturonic acid); ▲, polygalacturonase activity (substrate was methoxylated polygalacturonic acid).

TABLE I. PROPERTIES OF PECTIN

Property	Pectin		
	from *C. unshiu* peel		from lemon (Commercial product)
	Extracted by ppase-B[c]	Extracted by acid[d]	
Ash (%)	ND	3.5	2.6
Relative viscosity of 1% solution	1.44	1.23	1.53
Methoxyl group[a]	8.80	9.13	9.24
Galacturonic acid[a] (%)	75.8	80.3	85.0
Esterified carboxyl group (%)	69.9	70.7	68.2
Neutral sugar[a] (%)	21.2	10.5	5.7
pH of 0.5% solution	3.50	4.34	3.96
Molecular weight $\times 10^{-3}$ [b]	113	50	102
Elementary analysis C (%)	40.56	38.27	40.86
H (%)	5.73	5.40	5.76
N (%)	0.66	0.41	0.80

[a] Values expressed on an ash- and moisture-free basis.
[b] Calculated by the equation of Smit and Bryant.[11]
[c] Reaction was done in a mixture (100 ml) containing 50 g (as dry weight) of *C. unshiu* peel and 1000 units of protopectinase-B, at 37°C, for 24 hr.
[d] Reaction was done in a mixture (100 ml) containing 50 g (as dry weight) of *C. unshiu* peel and 100 μmol of H_2SO_4 at 60°C for 30 min.

procedure, 42 mg of purified enzyme was obtained with the overall yield of about 20% of the starting material. The purified enzyme was homogeneous by the criteria of electrophoreses in the presence or the absence of sodium dodecyl sulfate (SDS) (Fig. 1), and its homogeneity was also confirmed by ultracentrifugal analysis (not shown). The molecular weight of the enzyme was about 30,000 by gel filtration on the same column of Toyopearl HW-55S, and its isoelectric point was pH 9.4. The enzyme acted on protopectin and released highly polymerized pectin, which had a molecular weight of about 110,000 (Table I), and did not catalyze the degradation of polygalacturonic acid (Fig. 2). In Table I, some properties of the pectin released from the peel of the mandarin orange (*Citrus unshiu*) by the action of the purified enzyme are listed. The pectin obtained had a higher molecular weight than that obtained by the conventional method. Because of these properties, we decided that the enzyme was a new kind of protopectinase, and named it protopectinase-B. The enzyme showed activity of neither polygalacturonase, amylase, xylanase, cellulase, nor protease, but it degraded soybean arabinogalactan that binds the pectin molecule and a cell-wall constituent. However, the mechanism is not known.

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