Production of Alkaline Enzymes by Alkalophilic Microorganisms

Part III. Alkaline Pectinase of Bacillus No. P-4-N

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Bacillus No. P-4-N isolated from soil produced an alkaline polygalacturonase (APGase) in alkaline media. The characteristic point of this microorganism was especially good growth in alkaline media, and that very poor growth was detected in neutral media such as nutrient broth. An important factor for the production of APGase was the addition of manganese to the medium containing sodium carbonate, which was also another important factor. The APGase of Bacillus No. P-4-N was purified by Sephadex G-100 and DEAE-cellulose columns followed by Sephadex G-200 gel filtration. The enzyme was most active at pH 10.0 to 10.5 and stable pH was about 6. The enzyme was heat stable; about 80% of the activity remained after heating at 80°C for 5 min. Calcium ion was effective to stimulate the activity and to stabilize the enzyme. The molecular weight estimated by Sephadex gel filtration method was $6 - 7 \times 10^4$. The enzyme was completely inactivated by EDTA, but not by urea, NaCl and PCMB. The enzyme split polygalacturonic acid at random and yielded galacturonic acid, digalacturonic acid and higher oligogalacturonic acids. If the enzyme is a single entity, it is a type of liquefying APGase.

In the previous papers of this series, it has been reported that a certain species of bacteria isolated from soil in Japan can grow in alkaline media containing high concentration of sodium carbonate or sodium bicarbonate. Bacillus No. 221 accumulates a large amount of alkaline protease having the optimum pH at 11.5. Bacillus No. A-40-2 produces an alkaline amylase which has the optimum pH at 10.5.

By the analogous method, a strain producing alkaline polygalacturonase (APGase) has been isolated.

This paper deals with the isolation and characterization of the strain, Bacillus No. P-4-N which produces APGase, and some properties of the enzyme are also discussed.

MATERIALS AND METHODS

Medium to isolate APGase-producing microorganisms. A pectin medium was used to isolate the bacteria producing APGase: pectin, 30 g; polypeptone, 5 g; yeast extract (Difco), 5 g; K$_2$HPO$_4$, 1 g; MgSO$_4$·7H$_2$O, 0.2 g; MnSO$_4$·7H$_2$O, 0.05 g; Na$_2$CO$_3$, 10 g and 1 liter of water. Sodium carbonate was sterilized separately and added to the medium. The pectin medium was solidified by the addition of agar (1.5% w/v), if necessary.

Isolation method of alkalophilic bacteria producing APGase. A small amount of soil was suspended in sterilized water and spread on the pectin agar plates. The plates were incubated at 37°C for 48 hr. Colonies isolated were inoculated into the pectin medium and incubated at 37°C for 3 days with continuous shaking. Pectinase activities of the culture fluid were measured at pH values of 7.0 and 10.0. Bacillus No. P-4-N was isolated as a potent producer for alkaline APGase from about 100 colonies.

Characterization and identification of bacteria. Morphological properties and taxonomic characteristics of the bacteria were investigated according to the method described in "Aerobic Sporeforming Bacteria" and "Bergey’s Manual of Determinative Bacteriology."
Unless stated otherwise, media used for the identification were supplemented with 1% Na₂CO₃.

Preparation of a crude enzyme solution. *Bacillus* No. P-4-N was grown aerobically at 37°C in the tested media. After 2 to 3 days of incubation with continuous shaking, cells were removed by centrifugation at 6,000 × g for 10 min and the supernatant fluid was examined as a crude enzyme solution.

Assay of APGase. The enzyme (0.01 ml) containing up to 20 mU of APGase in 0.01 M Tris-HCl buffer (pH 7.0) was mixed with 0.2 ml of 1% pectic acid containing 1 mM CaCl₂ (made up with 0.05 M glycine-NaOH-NaCl buffer of pH 10.0). After 10 min incubation at 40°C, 1 ml of dinitrosalicylic acid solutions was added, and the mixture was heated in a boiling water-bath for 5 min, and then 4 ml of water were added to it. After centrifugation at 1,000 × g for 10 min, an optical density of the supernatant fluid was measured at 510 nm. One unit of the enzyme activity is defined as the amount of enzyme which produces one mg of reducing sugar calculated as galacturonic acid per minute under the above condition.

Measurement of viscosity. Viscosimetric measurements were carried out in Ostwald viscosimeters. Pectic acid solution containing 1 mM CaCl₂ (0.05 M glycine buffer, pH 9.0) was mixed with the enzyme and the enzymatic action was expressed as per cent viscosity change. This is equivalent to the Abbauzahl Λ of Weber and Deuel which is calculated as follows:

\[ \Lambda = V_0 - V_1 / V_0 - V_s \times 100 \]

where:
- \( V_0 \) = flow time in seconds of pectic acid + inactivated enzyme
- \( V_1 \) = flow time in seconds at reaction time of pectic acid + active enzyme
- \( V_s \) = flow time in seconds of the buffer + inactivated enzyme.

To inactivate the enzyme, the enzyme solution was heated at 100°C for 10 min.

Assay of pectinesterase activity. Five ml of 1.0% pectin solution were mixed with 0.1 ml of test solution (5 U in APGase) at pH 10.0, and the increase in acidity of the reaction mixture at 40°C was measured using a Hiranuma RAT 101 automatic titrator.

Assay of trans-eliminase activities toward pectin and pectic acid. Three ml of 1.0% pectin solution (pH 10.0, glycine buffer, 0.05 m) and 0.05 ml of test solution (2.5 U in APGase) were mixed and an absorbancy at 235 mλ was measured by a Hitachi double beam spectrophotometer at room temperature for 2 hr. Polygalacturonic acid trans-eliminase was measured in the similar way except that pectic acid was used as the substrate instead of pectin.

Protein concentration. Protein concentration was estimated by the method of Warburg and Christian.

Identification of enzymatic digest of pectic acid. The hydrolyzate was chromatographed on Whatman No. 1 paper with the solvent system of butanol-acetic acid-water (2:1:1 v/v) at room temperature. Ammoniacal silver nitrate was used as a spraying reagent.

Reagents. Pectin from citrus and pectic acid were obtained from Wako Pure Chemical Co., Ltd, Osaka.

RESULTS

Characters of *Bacillus* No. P-4-N

The isolated strain No. P-4-N was an aerobic, sporeforming, gram positive, motile, rod-shaped bacterium, and peritrichous flagella. It is clear that the bacterium should belong to the genus *Bacillus*. The characteristic point of the bacteria was that it grew well in alkaline media rather than neutral one such as nutrient broth, the optimal pH for growth was about 10.

Table I summarizes the morphological and cultural characteristics of the strain No. P-4-N. Taxonomic position of this strain will be reported elsewhere.

Culture conditions for production of APGase

Culture conditions for APGase production were examined in the media containing various amounts of sodium carbonate. Requirement of minerals was also tested. APGase activity after 48 hr³ cultivation was assayed. The results are shown in Table II, which indicates that the addition of carbonate and manganese salts is an important factor for the production of the APGase. The formation of APGase was adaptive, because no activity of APGase...
Production of Alkaline Enzymes by Alkalophilic Microorganisms. Part III

TABLE I. MORPHOLOGICAL, CULTURAL, AND BIOCHEMICAL CHARACTERISTICS OF STRAIN P-4-N

<table>
<thead>
<tr>
<th>1. Morphological characteristics</th>
<th>Growth</th>
<th>Glucose-nitrate agar slant</th>
<th>Glucose-asparagine agar slant</th>
<th>Anaerobic growth in glucose broth</th>
<th>Anaerobic production of gas from nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form</td>
<td>Rods</td>
<td>±</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Size</td>
<td>0.4–0.5×2–2.5 µ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram stain</td>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporangia</td>
<td>Definitely swollen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spores</td>
<td>0.7–0.9×1–1.2 µ; oval; subterminal</td>
<td></td>
<td></td>
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2. Cultural characteristics

<table>
<thead>
<tr>
<th>Nutrient broth</th>
<th>pH 7</th>
<th>pH 10.3</th>
<th>+</th>
<th>±</th>
<th>+</th>
<th>#</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient agar slant</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-nutrient broth</td>
<td>+</td>
<td></td>
<td>#</td>
<td>#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-nutrient agar slant</td>
<td>+</td>
<td></td>
<td>#</td>
<td>#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato</td>
<td>±</td>
<td></td>
<td>#</td>
<td>#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-medium(^\text{a}))</td>
<td>±</td>
<td></td>
<td>#</td>
<td>#</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Biochemical characteristics\(^a)\)

- Hydrolysis of gelatin and casein: Positive
- Hydrolysis of starch: Positive
- Utilization of citrate: Not utilized
- Utilization of ammonium salts: Utilized
- Reduction of nitrate to nitrite: Reduced
- Voges-Proskauer test: Not clear

4. pH and temperature for growth

- pH for growth in I-medium\(^b)\) pH 7 to pH 11
- Temperature for growth in I-medium, up to 55°C at pH 10.3.

\(^a\) Na\(_2\)CO\(_3\) (1%) was added to the media.

\(^b\) pH in the I-medium was adjusted by adding HCl or NaOH.

=, indicates no growth; ±, very poor growth; +, poor growth; #, normal growth; ##, growth abundant.

TABLE II. CULTURE CONDITIONS FOR PRODUCTION OF APGASE

*Bacillus* No. P-4-N was grown aerobically at 37°C in the tested media. After 2 days incubation with shaking, APGase activity was measured. MnSO\(_4\)·7H\(_2\)O is expressed as Mn\(^{2+}\); FeSO\(_4\)·7H\(_2\)O, Fe\(^{2+}\); CaCl\(_2\)·2H\(_2\)O, Ca\(^{2+}\).

<table>
<thead>
<tr>
<th>Pectin (%)</th>
<th>Peptone (%)</th>
<th>Na(_2)CO(_3) (%)</th>
<th>Initial pH</th>
<th>Mn(^{2+}) (%)</th>
<th>Fe(^{2+}) (%)</th>
<th>Ca(^{2+}) (%)</th>
<th>APGase (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>0</td>
<td>7.2</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0</td>
<td>7.2</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1</td>
<td>10.2</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>10.2</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>10.2</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1</td>
<td>10.2</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>7.2</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1.5</td>
<td>10.4</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>8.5</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>2.0</td>
<td>10.6</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>2.5</td>
<td>10.7</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1.5</td>
<td>10.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1.5</td>
<td>10.4</td>
<td>0.01</td>
<td>0</td>
<td>0</td>
<td>5.5</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1.5</td>
<td>10.4</td>
<td>0.001</td>
<td>0</td>
<td>0</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1.5</td>
<td>10.4</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>8.0</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1.5</td>
<td>10.4</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>6.0</td>
</tr>
</tbody>
</table>

(less than 0.2 U/ml) was detected in media containing glucose or starch instead of pectin.

**Purification of the enzyme**

*Bacillus* No. P-4-N was grown aerobically at 37°C in the pectin medium described above.
After 2 days' cultivation with shaking, the culture fluid (1,000 ml) was centrifuged at 6,000×g for 10 min. The supernatant fluid (6.5 U/ml) was divided into dialysis bags and concentrated by dipping in polyethylene glycol No. 6,000. The concentrates (30 ml) were passed through a Sephadex G-100 column (2.5×45 cm) which was equilibrated with 0.01 M phosphate buffer (pH 7.6). A typical elution profile is shown in Fig. 1. The active fractions were collected and loaded onto a DEAE-cellulose column (3×30 cm) equilibrated with 0.01 M phosphate buffer (pH 7.6). After the column was successively washed with 0.01 M phosphate buffer (pH 7.6) containing 0.1 M NaCl, the APGase was eluted with a linear gradient increase in NaCl at flow rate of 50 ml/hr. A linear gradient was obtained by using 400 ml each of 0.1 M and 0.4 M NaCl in 0.01 M phosphate buffer (pH 7.6); 7 ml fractions were collected and each fraction was assayed. A typical chromatogram is shown in Fig. 2. Fractions containing APGase were collected.

### Table III. Purification of APGase

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg protein)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture fluid</td>
<td>1,000</td>
<td>6.5</td>
<td>6,500</td>
<td>2,100</td>
<td>3.1</td>
<td>100</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>60</td>
<td>85</td>
<td>5,100</td>
<td>164</td>
<td>33.1</td>
<td>80</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>70</td>
<td>46.5</td>
<td>3,250</td>
<td>9.3</td>
<td>350</td>
<td>50</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>55</td>
<td>47.2</td>
<td>2,600</td>
<td>2.7</td>
<td>950</td>
<td>40</td>
</tr>
</tbody>
</table>
and concentrated by polyethylene glycol method mentioned above. The enzyme preparation (7 ml) was passed through a Sephadex G-200 column (2.5 x 45 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). Figure 3 shows a typical elution profile of the enzyme. The APGase was purified about 300-fold by these treatments. The results are shown in Table III. The following experiments were carried out by using this preparation.

Molecular weight and ultraviolet absorption spectrum

An estimation of the molecular weight of the enzyme was made by using a calibrated Sephadex G-100 column 1) (1.6 x 65 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl. On the column, 1 ml of purified APGase (20 U) was applied, and the flow rate was 35 ml/hr. On the assumption that the APGase conforms to linear relationship between the elution volume and the log of the molecular weight, the molecular weight is approximately 6.7 x 10^4. Ultraviolet absorption spectrum of the enzyme showed a typical UV absorption of protein. The ratio of absorption at 280 mÅ to 260 mÅ was about 1.4.

Effect of CaCl₂ on activity of APGase, and relation of enzyme concentration and reaction rate

The enzyme (15 mU) was mixed with 0.2 ml of 1 % pectic acid (0.05 M glycine-NaOH-NaCl, pH 10.0) containing various concentrations of CaCl₂ and enzyme activity was measured. As shown in Fig. 4, maximal activity was observed in 1 mM. Only 10% of activity was detected in the absence of CaCl₂. Thus, routine assays were always made in the reaction mixture with 1 mM CaCl₂. Various concentrations of the enzyme were mixed with 0.2 ml of 1 % pectic acid (0.05 M glycine buffer, pH 10.0) containing 1 mM CaCl₂. As shown in Fig. 5, under the condition of the assay described above, a straight-line relationship was maintained between reducing power and enzyme concentration (up to 20 mU).

![Fig. 4. Effect of CaCl₂ on APGase Activity.](image)

APGase (15 mU) was mixed with 0.2 ml of 1 % pectic acid (pH 10.0) containing indicated amounts of CaCl₂. The maximum activity is expressed as 100%.

![Fig. 5. Relation of Enzyme Concentration and Reaction Rate.](image)

Time course of hydrolysis

About 0.2 U of the enzyme (0.15 ml) was mixed with 3 ml of 1 % pectic acid or pectin containing 1 mM CaCl₂ (pH 10.0, 0.05 M glycine buffer). Aliquots (0.2 ml) of the reaction mixture were periodically taken, and reducing sugars produced were determined.
About 0.2 U of the enzyme (0.15 ml) was mixed with 3 ml of pectic acid or pectin solution of pH 10.0 and incubated at 40°C. Open circle, pectin; closed circle, pectic acid.

As shown in Fig. 6, maximum degree of hydrolysis was about 35%. Addition of the enzyme (0.2 U) did not cause further liberation of reducing sugar. Pectin was also hydrolyzed with the enzyme, although the velocity of hydrolysis was almost 50% of that of pectic acid.

**Effects of pH on activity and on stability of the enzyme**

Figure 7 shows effect of pH on APGase activity. The enzyme is most active at pH 10.0. Stability of the enzyme was investigated in buffer solution of various pH values containing 1 mM CaCl₂. The mixture was incubated at 70°C for 10 min, and the residual activity was determined at pH 10.0. As shown in Fig. 8, the enzyme is most stable at pH 6.5.

**Temperature optimum for enzyme action and thermal stability**

The optimum temperature was determined by varying the reaction temperature. In Fig. 9, the activity at 40°C was expressed as 100%. The activity at 65°C was about 70%. To examine the thermal stability, the enzyme was dissolved in 0.05 M Tris-maleate buffer (pH 6.5), and the solution was heated at the
Reaction was carried out at pH 10.0 for 10 min in the presence of 1 mM Ca²⁺.

Open circle, in the presence of Ca²⁺; closed circle, in the absence of Ca²⁺. See the text for the experimental details.

indicated temperatures for 10 min, the residual activity being measured at pH 10.0. Incubation was done in the presence or absence of Ca²⁺ (5 mM). As shown in Fig. 10, the enzyme was stable up to 70°C in the presence of Ca²⁺.

Effect of inhibitors
The enzyme was incubated with the reagents indicated in 0.05 M glycine buffer (pH 10.0) at 30°C for 30 min and the residual activity was determined. The enzyme was completely inactivated by 10⁻⁴ M of EDTA (ethylenediaminetetraacetic acid). Urea (8 M), 0.3 M of NaCl and 10⁻⁴ M of PCMB (p-chloromercuribenzoate) did not show any inhibitory effect.

Reduction in viscosity of pectic acid by the enzyme
The experiment was done at pH 9.0, because the viscosity at pH 10.0 was too low to determine. To 5 ml of 4% pectic acid solution containing 1 mM CaCl₂ (made up with 0.05 M glycine buffer, pH 9.0), 0.1 ml of the enzyme (0.1 U) was added. Decrease in viscosity and increase in reducing power by the enzyme are shown in Fig. 11. After 10 min incubation, about 50% of viscosity decreased, but hydrolysis degree which was calculated by reducing sugars liberated was only 2.5%.

Mode of action of the enzyme on pectic acid
About 10 mg of pectic acid were dissolved in 1 ml of water containing 1 mM CaCl₂ and
FIG. 12. Mode of Action of the Enzyme on Pectic Acid.

About 11 U of the enzyme were added to 1 ml of 1% pectic acid solution (pH 10.0, adjusted with NH₄OH), and incubated at 40°C. The hydrolyzates were chromatographed on Whatman No. 1 paper, and sprayed with ammoniacal silver nitrate. Its pH was adjusted to 10.0 with NH₄OH. To the pectic acid solution was added 0.1 ml of the enzyme (11 U) and the mixture was incubated at 40°C. Aliquots of the reaction mixture were periodically withdrawn and chromatographed on Whatman No. 1 paper using the solvent system of butanal-acetic acid-water (2:1:1 v/v). The reducing sugars were detected by spraying with ammoniacal silver nitrate. The results are shown in Fig. 12, indicating the liberation of di-, tri- and tetragalacturonic acids in ten minutes incubation. After 2 hr, tetragalacturonic acid disappeared and the accumulation of galacturonic acid was observed concomitantly.

Other enzymatic activities of the purified enzyme

Pectinesterase, pectin trans-eliminase and polygalacturonic acid trans-eliminase were not detected under the tested conditions.

DISCUSSION

The strain of Bacillus No. P-4-N isolated from soil produced new APGase in alkaline media. This strain grows well in alkaline media rather than neutral media. The morphological and cultural characteristics of this strain will be compared with other strains including other alkalophilic bacteria reported previously. The APGase was produced only in alkaline media containing pectin; the formation of the enzyme seems to be inducible. Manganese was an important factor to stimulate the enzyme production, although no remarkable change was observed in growth of the bacteria. The APGase was partially purified by Sephadex G-100 gel filtration and DEAE-cellulose chromatography, followed by Sephadex G-200 gel filtration. It is not clear whether the purified APGase is a homogeneous protein or not, because ultracentrifugal analysis, free-boundary electrophoresis, and disc electrophoresis, and disc electrophoresis have not yet been done. Endo reported that endo-polygalacturonase of Coniothyrium diploidiella was a mixture of three endo-polygalacturonases. There is a possibility that the purified APGase is a mixture of two or more enzymes. Further works on these points are now under investigation. The enzyme is active only in the presence of Ca²⁺; only 10% of activity was detected in the absence of Ca²⁺ and EDTA showed 100% inhibition. Calcium ion also has an effect on stability of the enzyme; the residual activity was about 100% when heated at 70°C in the presence of 5 mM CaCl₂ and 60°C in the absence of CaCl₂. Polygalacturonases of C. diploidiella were completely inactivated by heating at 60°C for 10 min. Clostridium felsineum polygalacturonase was somewhat thermostable; about 12% of activity remained after being heated at 60°C for 20 min. Therefore, the APGase seems to be thermostable. The enzyme has optimum pH at 10 toward pectic acid. This value is higher than those of polygalacturonases so far reported.

Deuel and Stutz classified polygalacturonase into three types, although every type may contain polygalacturonases of different specificities and properties. Type 1, the
liquefying polygalacturonases, which split the glycosidic linkages more or less at random. They preferentially attack pectins of low degree of esterification. Type 2, they preferentially attack pectins of high degree of esterification. Type 3, saccharifying enzyme, which hydrolyzes pectins only from one end of the chain molecule. If the purified enzyme is a single entity, the APGase of *Bacillus* No. P-4-N seems to belong to a type 1, a type of liquefying polygalacturonase.

As the results, I wish to conclude that the APGase is different from the polygalacturonases so far reported, especially in optimum pH.

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