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 10~10.5 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 $6\sim7\times 10^4$. 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,^{1,2)} 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 11.5.¹⁾ *Bacillus* No. A-40-2 produces an alkaline amylase which has the optimum pH at 10.5.²⁾

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; K₂HPO₄, 1 g; MgSO₄·7H₂O, 0.2 g; MnSO₄·7H₂O, 0.05 g; Na₂CO₃, 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"³⁾ and "Bergey's Manual of Determinative Bacteriology."⁴⁾

Unless stated otherwise, media used for the identification were supplemented with 1% Na_2CO_3 .

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 $6,000 \times g$ 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 CaCl_2 (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 solution⁵⁾ 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 $1,000 \times g$ for 10 min, an optical density of the supernatant fluid was measured at 510 m μ . 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 CaCl_2 (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 Deuel⁶⁾ which is calculated as follows:

$$A = V_0 - V_t / V_0 - V_s \times 100$$

V_0 = flow time in seconds of pectic acid + inactivated enzyme

V_t = 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 m μ 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.⁷⁾

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 hr' 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

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

1. Morphological characteristics	Glucose-nitrate agar slant	±	+
Form	Rods	±	+
Size	0.4~0.5×2~2.5 μ	—	—
Motility	Motile	—	—
Gram stain	Positive	—	—
Sporangia	Definitely swollen	±	+
Spores	0.7~0.9×1~1.2 μ; oval; subterminal		
2. Cultural characteristics	3. Biochemical characteristics ^{a)}		
	Hydrolysis of gelatine 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_2CO_3 (1%) was added to the media.

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

-, indicates no growth; $\pm$, very poor growth; +, poor growth; $\pm\pm$, normal growth; $\pm\pm\pm$, 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. $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ is expressed as Mn^{2+} ; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, Fe^{2+} ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Ca^{2+} .

Pectin (%)	Peptone (%)	Na ₂ CO ₃ (%)	Initial pH	Mn ²⁺ (%)	Fe ³⁺ (%)	Ca ²⁺ (%)	APGase (U)
1	0.5	0	7.2	0.005	0	0	0.2
2	0.5	0	7.2	0.005	0	0	0.2
3	0.5	0	7.2	0.005	0	0	0.2
1	0.5	1	10.2	0.005	0	0	0.5
2	0.5	1	10.2	0.005	0	0	4.5
3	0.5	1	10.2	0.005	0	0	7.2
3	0.5	1.5	10.4	0.005	0	0	8.5
3	0.5	2.0	10.6	0.005	0	0	2.3
3	0.5	2.5	10.7	0.005	0	0	1.5
3	0.5	1.5	10.4	0	0	0	2.1
3	0.5	1.5	10.4	0.01	0	0	5.5
3	0.5	1.5	10.4	0.001	0	0	5.0
3	0.5	1.5	10.4	0.005	0.005	0	8.0
3	0.5	1.5	10.4	0.005	0.005	0.005	6.0

(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.

FIG. 1. Column Chromatogram on Sephadex G-100.

Column, 2.5×45 cm; flow rate, 35 ml/hr; O.D. at $280\text{ m}\mu$ was monitored by an Ohtake UV analyzer (dotted line); open circle, APGase activity.

After 2 days' cultivation with shaking, the culture fluid (1,000 ml) was centrifuged at $6,000 \times g$ 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

FIG. 2. Column Chromatogram on DEAE-cellulose.

Column, 3×30 cm; load, about 160 mg; flow rate, 60 ml/hr; O.D. at $280\text{ m}\mu$ was monitored by an Ohtake UV analyzer (dotted line); closed circle, APGase activity; solid line, concentration of NaCl.

FIG. 3. Sephadex G-200 Column Chromatogram of Partially Purified Enzyme.

Column, 2.5×45 cm; flow rate, 30 ml/hr; open circle, APGase activity; dotted line, O.D. at $280\text{ m}\mu$.

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

	Volume (ml)	Activity (U/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)
Culture fluid	1,000	6.5	6,500	2,100	3.1	100
Sephadex G-100	60	85	5,100	164	33.1	80
DEAE-cellulose	70	46.5	3,250	9.3	350	50
Sephadex G-200	55	47.2	2,600	2.7	950	40

and concentrated by polyethylene glycol method mentioned above. The enzyme preparation (7 ml) was passed through a Sephadex G-200 column (2.5×45 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 column⁸⁾ (1.6×65 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 $6\sim7\times10^4$. Ultraviolet absorption spectrum of the enzyme showed a typical UV absorption of protein. The ratio of absorption at 280 m μ to 260 m μ 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.

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.

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.

FIG. 6. Time Course of Hydrolysis of Pectin and Pectic Acid.

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 CaCl_2 . 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

FIG. 7. Optimum pH of APGase.

The pH was adjusted with 0.1 M Tris-maleate buffer (pH 5~8.5), 0.1 M glycine-NaOH-NaCl buffer (pH 8.5~11.5) and 0.1 M Na_2CO_3 -NaOH buffer (pH 11.5~12).

FIG. 8. Effect of pH on Stability.

The following buffer systems were used: 0.05 M Tris-maleate (pH 5~8.5) and 0.05 M glycine-NaOH-NaCl (pH 9~11). The reaction mixture was incubated at 70°C for 10 min and the residual activity 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 700%. To examine the thermal stability, the enzyme was dissolved in 0.05% Tris-maleate buffer (pH 6.5), and the solution was heated at the

FIG. 9. Optimum Temperature for Enzyme Action.

Reaction was carried out at pH 10.0 for 10 min in the presence of 1 mM Ca^{2+} .

FIG. 10. Thermal Stability of the Enzyme.

Open circle, in the presence of Ca^{2+} ; closed circle, in the absence of Ca^{2+} . 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 Ca^{2+} (5 mM). As shown in Fig. 10, the enzyme was stable up to 70°C in the presence of Ca^{2+}

Effect of inhibitors

The enzyme was incubated with the reagents

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^{-2} M of EDTA (ethylenediamine-tetraacetic 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

FIG. 11. Decrease in Viscosity and Increase in Reducing Power of Pectic Acid Solution by Incubation with the Enzyme.

Pectic acid solution (5 ml, 4%, pH 9.0) was hydrolyzed with 0.1 ml of the enzyme (0.1 U). See the text for further details. Open circle, viscosity; closed circle, hydrolysis rate.

containing 1 mM CaCl_2 (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 CaCl_2 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 NH_4OH), and incubated at 40°C . The hydrolyzates were chromatographed on Whatman No. 1 Papers, and sprayed with ammoniacal silver nitrate.

its pH was adjusted to 10.0 with NH_4OH . To the pectic acid solution was added to 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.^{1,21} 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 endopolygalacturonase of *Coniothyrium diplodiella*⁹¹ was a mixture of three endopolygalacturonases. 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 Ca^{2+} ; only 10% of activity was detected in the absence of Ca^{2+} 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 CaCl_2 , and 60°C in the absence of CaCl_2 . Polygalacturonases of *C. diplodiella*^{9,101} were completely inactivated by heating at 60°C for 10 min. *Clostridium felsineum*¹¹¹ 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 Stutz¹²¹ 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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