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Biochemical Properties of Starch Granule Non-Digestive Enzyme(SGNA) of *Bacillus polymyxa* No.26

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A α-1,4-D-glucan maltohydrolase (β-amylase), secreted by the mesophilic aerobic bacterium *Bacillus polymyxa* No.26, was purified and characterized. The enzyme production was increased after a logarithmic phase of bacterial growth and paralleled with the onset of bacterial sporulation. By applying anion exchange chromatography and gel filtration the enzyme was purified 16.7-fold and had a specific activity of 285.7 units/mg. Two enzyme activities were eluted on a column of DEAE-Sephadex chromatography, and they were designated as E-I for a major enzyme peak and E-II for a minor peak. Of them, E-I enzyme peak was further purified by using gel chromatography. The molecular mass of this enzyme was determined to be 64,000 daltons and consisted of a single subunit, showing an isoelectric point of 8.9. The enzyme was able to attack specifically the α-1,4-glycosidic linkages in soluble starch and caused its complete hydrolysis to maltose and β-limited dextrin. This amylolytic enzyme displayed a temperature optimum at 45°C and a pH optimum at 7.0. The amino acid composition of the purified enzyme was quite similar to the other bacterial β-amylases reported. Surprisingly, the purified enzyme from this aerobe only exhibited hydrolytic activity on soluble starch, not on starch granules. The degradation of raw starch by β-amylase was greatly stimulated by pullulanase addition. These results differentiated from other β-amylases reported. Based on a previous result that showed the enzyme system involves in effective degradation of raw starch granules, this result strongly suggested that the purified enzyme (E-I) can be a synergistic part of starch granule-digestion and E-II plays a crucial role in digestion of starch granules.

β-Amylase(EC 3.2.1.2, α-1,4-D-glucan maltohydrolase, saccharogenic amylase) is an exo-acting carbohydrase which cleaves alternative α-1,4-glucosidic linkages of starch from the nonreducing end producing β-maltose (2). It is a commercially important enzyme useful for food and beverage industries. β-Amylase occurs widely in many higher plants and is also produced by microorganisms (15). It has been generally accepted that bacterial β-amylases are adsorbed on to starch granules and degrade it to some extent (12,14), even though the starch granule-degrading capacity can not be compared to other raw starch degrading α-amylases

In a previous paper (16), we reported the starch granule-degrading β-amylase system for industrial application. The bacterial strain, isolated and identified as *Bacillus polymyxa* No.26, produced a large amount of β-amylase which can effectively breakdown starch granules. The bacterium produced 130 units of β-amylase per ml in the induction medium consisting of dextrin as the carbon source. This value of enzymatic activity was compared to other β-amylases of bacterial origin. In this communication, we report on the purification of starch granule non-digestive β-amylase(SGNA) and enzymatic properties.

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Key words: β-Amylase, sporulation, synergistic action, β-maltose, *Bacillus polymyxa* No. 26

MATERIALS AND METHODS

Cultivation of the Organism and Preparation of Enzyme

The β-amylase-hyperproducing strain No.26 was used (16). The culture was grown aerobically in an induction medium containing 3% dextrin as carbon source at 40℃ for 3 days (16). The culture broth containing cells was concentrated and the cells were separated by centrifugation (15,000 g, 30 min). Ammonium sulfate was added dropwise to the concentrated supernatant solution while stirring to give a final ammonium sulfate concentration of 75% (w/v), and this solution was left overnight at 4℃. The precipitate formed was collected by centrifugation (15,000 g, 30 min), dissolved in phosphate buffer (50 mM, pH 6.0), and dialyzed against the same buffer. The dialysed enzyme solution was used as the crude enzyme.

Enzyme Assay

The β-amylase activity was assayed as follows. The reaction mixture (1 ml) containing boiled soluble starch (2%), phosphate buffer(50 mM, pH 6.0) and appropriately diluted enzyme solution was incubated at 40℃ for 10 min. The reducing sugars liberated were measured by the dinitrosalicylic acid method (6). One unit of β-amylase was defined as the amount of enzyme that produced 1 μmole reducing sugar as maltose/min under the above conditions.

Pullulanase(from *Mycrococcus* sp. Y-1 described by Kim, C-H *et al.*) (5) activity was assayed in a reaction mixture (1 ml) that contained pullulan (2%), phosphate buffer (pH 6.0) and the enzyme solution. After a 10 min reaction at 40℃, the amount of reducing sugar formed was determined by dinitrosalicylic acid (DNS) method. One unit of pullulanase activity was that amount of enzyme that produces 1 μmole reducing sugar with glucose as standard per min under the conditions described.

Starch Granule Adsorption and Desorption

Raw corn starch (200 mg) was washed with phosphate buffer (2 ml, 50 mM, pH 6.0). 2 ml of enzyme solution at 4℃ and the mixture was kept for 15 min with occasional shaking. After centrifugation (10,000 g, 5 min), β-amylase activity of the supernatant solution was assayed and the adsorption percentage was calculated. The residue was washed twice with phosphate buffer (2 ml, 50 mM, pH 6.0). It was then suspended in elutant solution (2 ml) containing boiled soluble starch in phosphate buffer (50 mM, pH 6.0, with 5 mM $CaCl_2$), shaken occasionally for 30 min and centrifuged. The supernatant solution was used to assay the desorbed enzyme activity.

Raw Starch and Cross Linked Potato(CLP) Starch Digestion

The reaction mixture (2.0 ml) containing raw starch (50 mg), phosphate buffer (50 mM, pH 6.0), enzyme solution and a few grains of toluene was incubated at 37℃ with occasional shaking. At suitable time intervals, reducing sugars liberated in the reaction mixture were determined by DNS method. Cross linked potato starch (DP=5) was manufactured using the method described (4).

DEAE-Sephadex A-50 and Sephadex G-100 Column Chromatography

Supernatant containing β-amylase was dissolved in 0.01 M phosphate buffer, pH 7.6, and put on a DEAE-Sephadex A-50 column (1.6 × 40 cm) previously equilibrated with 0.01 M phosphate buffer, pH 7.6. Fractions (0.5 ml) of β-amylase were collected separately and lyophilized.

As a further experiment, the upper enzyme preparation was dissolved in 0.02 M phosphate buffer solution (pH 7.6) and put on a Sephadex G100 column (1.6 × 70 cm) previously equilibrated with the same buffer. Fractions (10 ml) were then eluted with the same buffer and used as purified enzyme solution.

Other Analytical Methods

Protein was estimated by the method of Lowry *et al.* with bovine serum albumin as standard (9). The isoelectric point was determined on a LKB Phast system containing carrier ampholine with a pH range of 3 to 9. Electrophoresis was performed with the potential gradient of 300 volts for 2 hr. Total carbohydrate was estimated by the phenol-sulfuric method (1). SDS-slab polyacrylamide gel electrophoresis was performed as described by Laemmli (8). The reaction products of β-amylase from soluble starch were analyzed by thin-layer chromatography as described in the previous report (16). Amino acid composition was analysed by HCl-hydrolysis method, except for tryptophan analysis, which was done as described (6).

RESULTS AND DISCUSSION

Morphological Changes during Microbial Cultivation and β-Amylase Production

Bacillus polymyxa No.26 can produce a novel β-amylase system which effectively degrades raw starch granules. The β-amylase productivity of this new strain was compared to other enzymes from other microorganisms reported (13, 17). These results were extensively disccused in a previous paper (16). Recently, It was found that the β-amylase production of *B. polymyxa* No.26 was highly related to the bacterial sporulation. The enz-

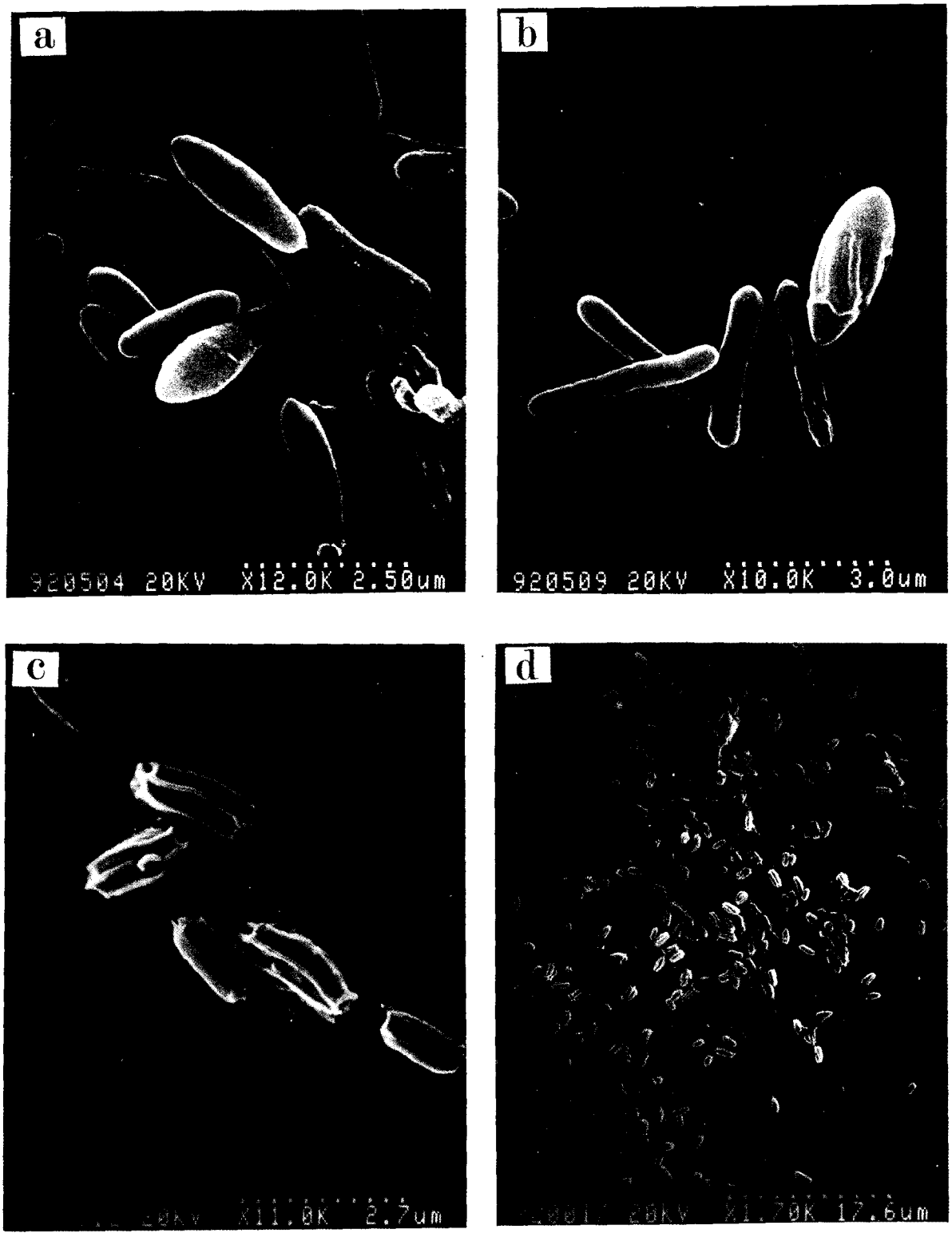

Fig. 1. Scanning electron microscopic (SEM) observation of the bacterial cells during β-amylase production.
a) after 1 day-growth; b) After 2-days growth; c) after 4-days growth; d) after 7-days growth. See text and our previous paper (5) for microscopic observation.

yme production was induced when the bacterial growth was reached in the stationary phase, and the enzyme production began to decrease with complete formation of the spore (16). To explain this phenomenon, whether the bacterial sporulation is correlated to the enzyme production or not was examined. As shown in Fig. 1, bacterial sporulation sensitively corresponded to the enzyme production, showing that the enzyme production of the 4-day growth was maximum (Fig. 1c). These results reasonably supported our previous findings that the enzyme production was controlled by the spore formation of bacterium. As shown in Fig. 3, the objective enzyme band displaying the enzyme activity was changed to a new band of a slightly small protein with concomitant increase of total enzyme activity (Fig. 3C). This result indicated that the protein modification by some proteolytic activity allowed the enzyme to be activated.

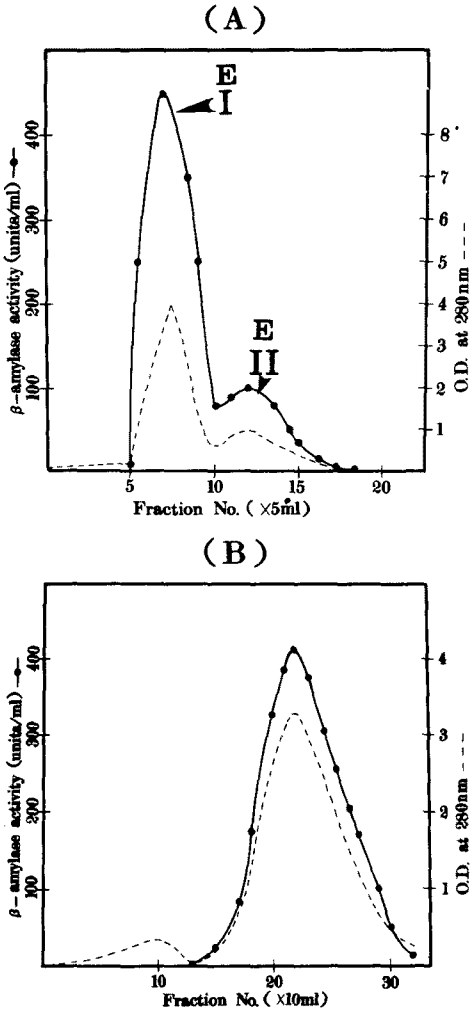

Fig. 2. Elution pattern of chromatographic purification of β-amylase.

A) Elution pattern of DEAE-Sephadex A-50 column chromatography. Symbols: Absorbance at 280 nm (---), enzyme activity (●). B) Elution pattern of Sephadex G-100 column chromatography. Symbols: Absorbance at 280 nm (---), enzyme activity (●).

Purification of Starch Granule Non-digestive β-Amylase(SGNA) as a Main Enzyme

After cultivation of *B. polymyxa* No.26 in batch culture, approximately 130 units/ml β-amylase was detected as extracellular protein in the culture supernatant. The specific activity of this enzyme was 17.1 units/mg. Analysis of the extracellular proteins using one-dimensional PAGE showed a protein band displaying β-amylase activity (Fig. 3B and C). Cell-free supernatant (1000 ml) of this culture broth after ammonium sulfate precipitation and fractionation by raw starch-adsorption was then applied to a DEAE-Sephadex A-50 ion exchange chromatography column, and the proteins were eluted with 10mM phosphate buffer, pH 7.6, at a flow rate of 0.5 ml/min. The fractions which were eluted during washing of the column contained impure proteins only. Two fractions, each containing β-amylase activity were then eluted. The major peak and minor peak shown in Fig.2A were eluted, and they were tentatively designated as E-I and E-II, respectively. The enzyme exhibiting

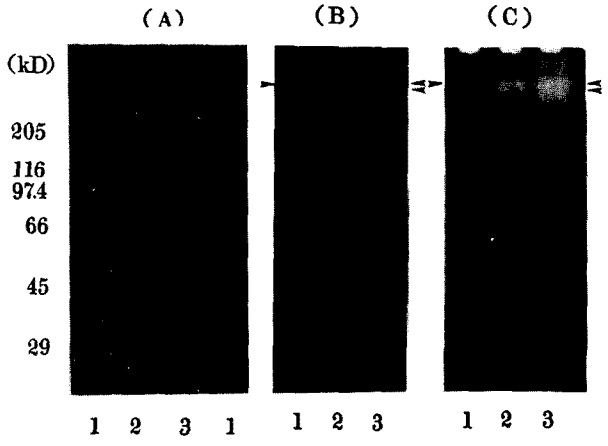

Fig. 3. Electrophoretic analysis of β-amylase preparations.

Slab gel electrophoresis was performed by the method described in Materials and Methods. A polyacrylamide gel (7.5 % for native-PAGE, 10% for SDS-PAGE) was used about 20 μg of samples were used and 2 mA per lane was applied for 120 min. Protein staining was done with 0.5% Coomassie brilliant blue R-250 and β-amylase zymogram was obtained by I_2-KI method described in Materials and Methods. A) SDS-PAGE. lane 1, molecular weight marker; lane 2, purified β-amylase; lane 3, crude enzyme solution. B) Native-PAGE. lane 1, crude enzyme solution after 2-days cultivation; lane 2, solution after 3-days cultivation; lane 3, solution after 4-days cultivation. C) Zymogram of native-PAGE (B). Lanes 1 to 3 correspond to numbers described in panel B).

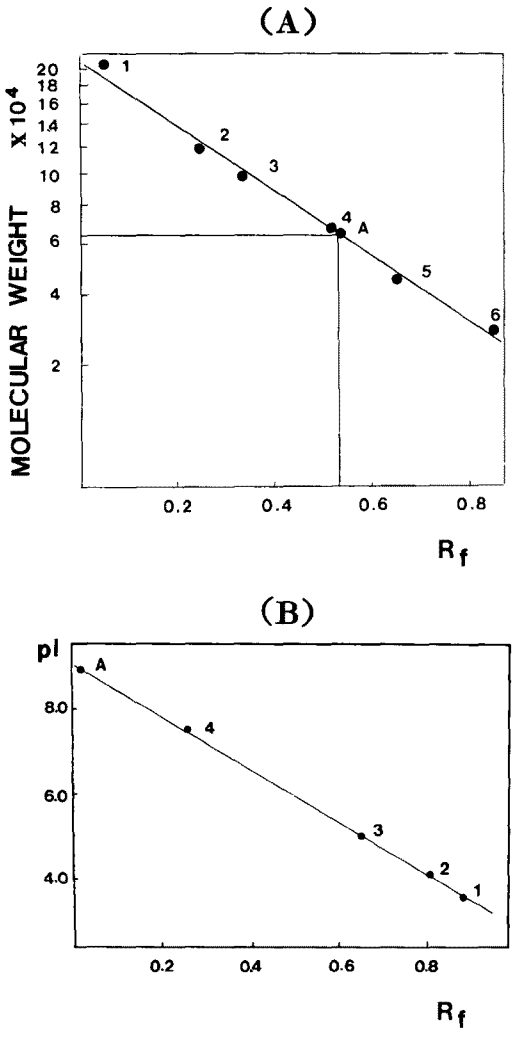

Fig. 4. Determination of molecular weight and isoelectric point of the purified β-amylase.

(A) Determination of the molecular weight by SDS-PAGE. ●, SDS-PAGE standard(high) of Sigma Co(SDS-6H); phosphorylase b(94,000), bovine serum albumin (66,000), ovalbumin (45,000), soybean trypsin inhibitor (22,000). (B) Determination of isoelectric point. ●, Isoelectric point standard maker (Sigma Co.); amyloglucosidase (pI 3.6),trypsin inhibitor (pI 4.6), β-lactoalbumin A (pI 5.1), carbonic anhydrase (pI 6.6). "A" denotes the purified β-amylase.

β-amylase activity present in the major peak was purified 14.6-fold with a recovery of 26.1%. The two enzyme sources of E-I and E-II was assayed for raw starch digestibility. Only E-II enzyme was active in hydrolyzing the raw starch, and E-I enzyme was active only to soluble starch. This result clearly indicated that the β-amylase system of this strain contains two β-amylase, one (E-II) for raw starch-digestion and the other (E-I) for soluble starch-digestion.

Further purification of this enzyme (E-I) in the major fraction was achieved by applying this sample to gel filtration column chromatography using the Sephadex G-100 column. One peak containing β-amylase activity (fractions 5 to 10) could be eluted after running the column at a flow rate of 0.5 ml/min (Fig. 2B). The final purified enzyme preparation (fractions 15 to 30) had a specific activity of 285 units/ml of β-amylase and was purified 16.7-fold with a yield 15.38%. The data of the purification procedure are shown in Table 1.

Criteria for Enzyme Purity, Molecular Weight and Isoelectric Point

Analysis of purified E-I fraction by SDS-PAGE revealed a single protein band (Fig. 3A). This purity was also confirmed by isoelectric foccusing. The purified enzyme showed its isoelectric point of 8.9 (Fig. 4B), indicating that the value is higher than those of other origins, including microorganisms and plants (3, 11, 13, 17). Using gel filtration, the molecular weight of the purified β-amylase was determined to be 64,000 daltons. In order to determine the subunit structure of β-amylase, the homogeneous enzyme was treated with SDS and 2-mercaptoethanol that was applied to SDS-PAGE. This resulted in the appearance in one band with a molecular mass of about 64,000 daltons (Fig. 3 and Fig. 4A). The native enzyme, therefore, consists of one subunit. The amino acid composition of the *B. polymyxa* No.26 β-amylase was analyzed by using acid- and alkali-hydrolysis method ascertaining the protein, and the result is shown in Table 2. The result was similar to those of other microbial β-amylases reported already, suggesting that the microbial enzyme sources are functionally related to each

Table 1. Purification of β-amylase from *Bacillus polymyxa* No.26.

Fraction	Total activity ($\times 10^4$ U)	Total protein ($\times 10^3$ mg)	Specific activity (U/mg)	Yield (%)	Fold
Cell-free culture broth	13.0	7.60	17.1	100	1
Raw starch-adsorption	11.2	2.2	50.1	94.2	2.9
Ammonium sulfate precipitate (75%)	9.5	0.65	146.1	73.1	8.5
DEAE Sephadex A-50	3.5	0.14	250.0	26.1	14.6
Sephadex G-100	2.0	0.07	285.7	15.38	16.7

Table 2. Amino acid composition of *Bacillus polymyxa* No.26 β-amylase.

Amino acid	Amino acid Mol %	No	Amino acid	Amino acid Mol %	No
Asx	11.27	54.4	Leu	5.81	28.35
Thr	6.78	36.43	Phe	0.33	1.28
Ser	9.12	55.44	His	8.29	34.19
Glu	7.44	32.36	Trp	8.13	25.48
Pro	5.32	29.57	Lys	6.39	27.97
Gly	11.66	99.41	Arg	1.55	5.69
Ala	9.72	69.83	Cys	0.42	1.59
Val	3.62	19.78	Met	trace*[1]	–
Ile	4.13	20.15	Tyr	trace*[1]	–

*[1] Only a trace amount of the amino acid was detected.

other (10). The molecular cloning of the β-amylase-encoding gene from the bacterium and its successful expression in *Bacillus subtilis* and other bacterial host systems is in progress. The results will be reported elsewhere.

Substrate Specificity and Effects of pH and Temperature on the Enzymatic Activity

Analysis of the fraction containing homogeneous protein showed that the purified enzyme possessed properties with the ability to attack α-1,4-glycosidic linkages. Soluble starch was almost completely hydrolyzed by the purified enzyme. The identification of the end-product of hydrolysis by TLC as maltose was achieved by applying pure maltose as standard (Fig. 5). Therefore, it was concluded that the enzyme was a β-amylase based either on the fact that the reaction product was maltose (Fig. 5) (16) or that iodine-staining color of soluble starch did not disappear during extensive hydrolysis (data not shown). In this report, maltose was found to be the only low molecular weight product from soluble starch and to be β-form. This result clearly indicates that the enzyme we purified is a β-amylase. The enzyme showed the maximum activity at pH 7.0 as shown in Fig. 6B. It had no activity at pH under 4.0 or over 9.5. The enzyme was stable from pH 5.0 to 8.0. The temperature-activity profile of the enzyme is shown in Fig. 6A. It showed the maximum activity at 45℃ and no activity over 60℃. These results indicate that optimum pH and temperature of the β-amylase obtained in this work are very close to those of other microbial enzymes (10-14). β-Amylase has been purified and studied from various higher plants such as soybean, barley, wheat, rice, and sweet potato. Some basic properties of *B. polymyxa* No.26. β-amylase have been compared to those of other higher plant and microbial β-amylases. *B. polymyxa* No.26 β-amylase has similar properties to those of other β-amylases in terms of optimum pH, pH stability, optimum temperature, temperature stability, molecular weight and amino acid composition. However, the most different feature of *B. polymyxa* No.26 β-amylase may be the high pI value.

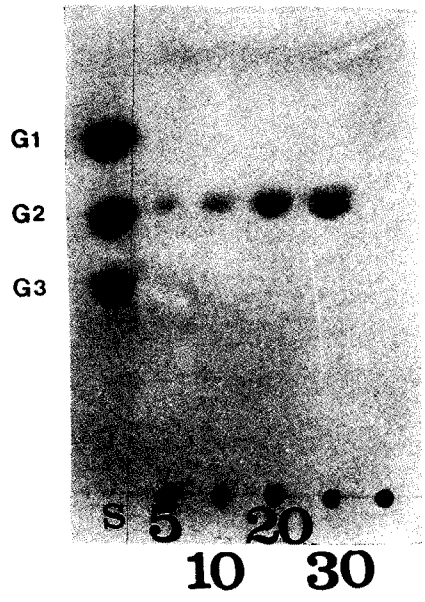

Fig. 5. TLC analysis of soluble starch-hydrolyzates by purified enzyme.

One-ml portions were withdrawn from a standard reaction mixture (10 ml) at various times and boiled to inactivate the enzyme. 10 μl each of these samples was spotted on a silica gel plate and thin layer chromatographed. Another portion of the boiled samples was examined for reducing sugars as described in a previous paper (5).

Synergistic Action of β-Amylase with Pullulanase on Raw Starch Granules

Even though the enzyme system of the donor bacterium was quitly active in utilizing insoluble raw substrates, the purified enzyme (E-I) was active only against soluble substrate, not against insoluble starch granules. This result allowed making the conclusion that the E-II enzyme plays a role in utilizing raw substrates. The rate of the combined action of β-amylase and pullulanase(from *Mycrococcus* sp. Y-1 described by Kim, C-H *et al.* (1992) (5) is shown in Table 3. The β-amylase alone could not attack raw starches (i.e., <1% hydrolysis under test conditions). Pullulanase clearly stimulated β-amylase hydrolysis activity on raw starch. Therefore, the effect of pullulanase concentration on raw corn starch digestion was further examined. The maximum stimulation (3.2 fold) occured at a pullulanase concentration of 4.7 units and a pullulanase to β-amylase ratio (unit: units) of 4. Further increasing the concentrations did not enhance hydrolysis activity.

To discover the raw starch-digesting β-amylase system of this strain, purification of E-II enzyme fraction and gene cloning of E-I and E-II genes are now in progress.

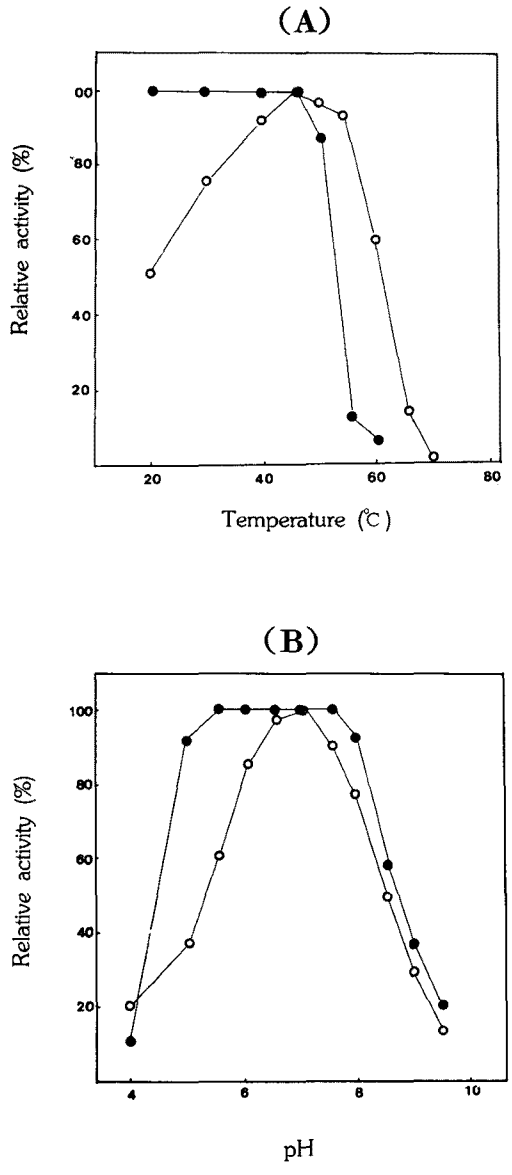

Fig. 6. Effects of temperature and pH on the enzyme activity of purified β-amylase.
(A) temperature-activity and stability profiles. For activity (○), the enzyme (0.1 ml) was incubated with 2% soluble starch in 0.01 M phosphate buffer (pH 6.0) at various temperatures and the reducing sugars were measured. For stability (●), the enzyme (0.1 ml) was incubated at various temperatures for 60 min and then the remaining activity was assayed at 30℃ for 60 min by the method described in Materials and Methods. (B) pH-activity and stability profiles. For activity (○), the enzyme was assayed under experimental conditions except that a McIlvaine buffer(pH3.0-9.0) was used. For stability (●), the enzyme (0.1 ml) was incubated in a McIlvaine buffer (pH 3.0~9.0) or Atkins-Pantin buffer (pH 8.0~10.0) at 30℃ for 60 min, and then the remaining activity was assayed under the standard conditions (pH 6.0).

Table 3. Comparison of raw starch hydrolysis by *Bacillus polymyxa* No.26 β-amylase in the presence and absence of pullulanase.

Starch source	Hydrolysis(%)[a]	
	β-amylase	β-amylase+pullulanase
Corn	0.82	18.5
Potato	0.34	6.2
CLP[b]	0.31	4.9

[a] The reaction mixture (2.0 ml) contained 50 mg raw starch, phosphate buffer (50 mM, pH 6.0) and β-amylase (3.0 units) with or without pullulanase (0.63 units, from *Mycrococcus* sp. Y-1) (5). Reaction time was 24 hr at 40℃.
[b] Cross linked potato starch described by Kim, C-H *et al.* (1990) (4).

These results will be reported upon completion of the experiments.

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