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Production and Characteristics of Raw-Potato-Starch-Digesting α-Amylase from *Bacillus subtilis* 65

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A newly isolated bacterium, identified as *Bacillus subtilis* 65, was found to produce raw-starch-digesting α-amylase. The electrophoretically homogeneous preparation of enzyme (molecular weight, 68,000) digested and solubilized raw corn starch to glucose and maltose with small amounts of maltooligosaccharides ranging from maltotriose to maltoheptaose. This enzyme was different from other amylases and could digest raw potato starch almost as fast as it could corn starch, but it showed no adsorbability onto any kind of raw starch at any pH. The mixed preparation with *Endomycopsis* glucoamylase synergistically digested raw potato starch to glucose at 30°C. The raw-potato-starch-digesting α-amylase showed strong digestibility to small substrates, which hydrolyzed maltotriose to maltose and glucose, and hydrolyzed *p*-nitrophenyl maltoside to *p*-nitrophenol and maltose, which is different from the capability of bacterial liquefying α-amylase.

As reported previously (9), a protease-negative mutant of *Aspergillus ficum*, M-33, produced a novel type of raw-starch-adsorbable, raw-starch-digesting α-amylase, α-amylase I (molecular weight, 88,000), but a hyperprotease mutant, M-72, from the same parental strain produced a conventional raw-starch-unadsorbable, raw-starch-nondigesting α-amylase, α-amylase II (molecular weight, 54,000). We therefore concluded that the fungal α-amylase was divided into two types: raw-starch-digesting α-amylase I and raw-starch-nondigesting α-amylase II. Thus, there are many fungal α-amylases and fungal glucoamylases (9). These results suggested that there might be two types of bacterial α-amylase as well, raw-starch-digesting and raw-starch-nondigesting. Here we describe the purification and characteristics of a novel type of raw-potato-starch-digesting bacterial α-amylase.

MATERIALS AND METHODS

Microorganisms. No less than 1,000 strains of bacteria were isolated from soils taken from near Fukuoka, Japan, by using raw-corn-starch-mineral-agar medium, which consisted of 2 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.7 g of K_2HPO_4, 0.3 g of KH_2PO_4, 20 g of agar, and 10 g of corn starch per 1,000 ml of distilled water. The corn starch was previously sterilized in an oven at 105°C for 2 h.

Chemicals. Potato starch (Japanese Pharmacopeia) was purchased from Maruishi Co., Ltd. Corn starch, sweet potato starch, and wheat starch were purchased from Wako Pure Chemical Co., Ltd. Soluble starch was purchased from Ishizu Pharmaceutical Co., Ltd. Waxy corn starch was prepared by the methods of Fujimoto et al. (3) and Sugimoto et al. (17). Glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7) were purchased from Nakarai Chemicals, Ltd. *p*-Nitrophenyl-α-D-glucopyranoside (PNP-G1) and *p*-nitrophenyl-α-D-maltoside (PNP-G2) were purchased from Sigma Chemical Co., Ltd. Liquefying α-amylase of *Bacillus subtilis* was purchased from Seikagaku Kogyo Co., Ltd.

Identification of bacteria. Morphological, physiological, and biochemical studies on the bacterium were carried out as described in *Bergey's Manual of Determinative Bacteriology* (1).

Bacterial culture. The isolated bacteria were routinely maintained on medium containing 10 g of beef extract (Difco Laboratories, Detroit, Mich.), 10 g of peptone, 10 g of G1, 5 g of NaCl, 15 g of agar, and 1,000 ml of distilled water. For seed cultures portions (1 liter) of nutrient broth, which consisted of 10 g of beef extract (Difco), 10 g of peptone, 10 g of soluble starch, 5 g of NaCl, and 1,000 ml of distilled water, were dispensed into 5-liter, round, flat-bottom flasks; autoclaved at 121°C for 20 min; and inoculated with bacteria from a freshly grown slope culture on a reciprocating shaker at 30°C for 24 h. For the main submerged culture, 120 liters of nutrient broth was sterilized at 121°C for 20 min in a 200-liter stainless steel fermentor (Marubishi Bioengineering Co., Ltd., Japan), inoculated with previously prepared seed culture (5%, vol/vol), cultivated at 30°C for 24 h with agitation at 200 to 250 rmp, and aerated at a rate of 1 volume of medium per minute.

Assay of α-amylase activity. α-Amylase activity was determined by incubating 1 ml of an appropriately diluted enzyme solution with 5 ml of 1% gelatinized potato starch solution, 1 ml of deionized water, and 1 ml of 0.1 M acetate buffer (pH 6.0) at 60°C. After 10 min, the reducing sugar that formed was determined by the micro-Bertrand method (13). One unit of α-amylase was defined as the amount of enzyme releasing 1 μmol of reducing sugar as the glucose standard from the substrate per min.

Determination of raw starch digestion. A reaction mixture containing 0.3 g of raw starch, 36 ml of deionized water, 6 ml of α-amylase solution (2.0 U/ml), 6 ml of 0.1 M acetate buffer (pH 6.0), and 1 ml of toluene was incubated at 30°C. At suitable intervals, the reducing sugar that formed in 1 ml of the reaction mixture was determined by the micro-Bertrand method (13), and the degree of hydrolysis was calculated.

Adsorbability of α-amylase activity to raw corn starch. The desired amount of α-amylase was added to buffers with various pHs to prepare an α-amylase solution of 2.0 U. Raw corn starch (1 g) was added to 5 ml of the prepared solution and left to stand at 4°C for 15 min. After centrifugation, the α-amylase activity of the supernatant was assayed and compared with that of the original α-amylase solution. The

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adsorption rate (AR) was defined by the following equation: AR (%) = $[(B - A)/B]100$.

Purification of α-amylase. *B. subtilis* 65 was cultivated and the resulting culture filtrate was collected for enzyme purification as described above. The following procedures were carried out at 4°C, unless stated otherwise.

(i) Step 1. Culture broth was centrifuged to remove bacterial cells, and $(NH_4)_2SO_4$ was added to a concentration of 20%. The suspension was left overnight and centrifuged at 10,000 × *g* for 30 min to remove precipitated proteins. Corn starch was added to the suspension to a concentration of 20%, and the suspension was stirred to adsorb α-amylase. The starch was washed 3 times with the same volume of 20% $(NH_4)_2SO_4$ solution in 0.14 M phosphate buffer (pH 6.1) by centrifugation. The amylase was eluted from the starch by stirring the solution in 1 liter of 0.05 M acetate buffer (pH 6.0) for 1 h at 30°C. The starch was subjected to elution 2 more times and the three eluates were combined. $(NH_4)_2SO_4$ was added to the combined eluates to a concentration of 50%, and the precipitated α-amylase was dissolved in 0.05 M Tris hydrochloride buffer (pH 8.0) and dialyzed overnight against the same buffer.

(ii) Step 2. After dialysis against 0.05 M Tris hydrochloride buffer (pH 8.0), the enzyme solution was applied to a Sephacryl S-300 column (2.5 by 100 cm) that was previously equilibrated with 0.05 M Tris hydrochloride buffer (pH 8.0).

(iii) Step 3. The α-amylase obtained by Sephacryl S-300 column chromatography was placed on a CM Sephadex C-50 column (2.3 by 57 cm) that was previously equilibrated with 0.05 M Tris hydrochloride buffer (pH 8.0), and the α-amylase was eluted with a linear NaCl concentration gradient from 0 to 1.0 M in the same buffer. The active fractions were pooled, concentrated, and used for the following experiments.

Electrophoresis. Disc gel electrophoresis was done by the method of Hendrick and Smith (10) with a 7.5% polyacrylamide gel column and a current of 2 mA per tube. Protein was stained with Coomassie brilliant blue R-250. The molecular weight of this enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Weber and Osborn (20).

Determination of molecular parameters. The sedimentation coefficient was determined with a centrifuge (model E; Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) equipped with an analytical rotor (An-D) and UV optics (Schlieren; Beckman) by the method of Martin and Ames (15). Enzyme protein (10 mg) was dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.6). The enzyme solution was dialyzed against the same buffer at 4°C for 48 h and applied to the cell. Centrifugation was done at 56,100 rpm and 20°C for 120 min. The Stokes radius was measured with Sephadex G-200 by the method of Siegel and Monty (16).

Effect of metal irons and chemical reagents. The purified amylase fraction was incubated with 2 mM metalic chloride, 0.1 mM EDTA, 0.2 mM chloromercuribenzoate, and 2 mM iodoacetamide (40°C, 30 min). The residual activities were determined under standard conditions.

Kinetic constants for various substrates. Kinetic constants for various substrates were measured by the methods of Horio and Yamanaka (11) and Lineweaver and Burk (14).

Paper chromatography. Sugar products were identified by paper chromatography with Whatman no. 1 filter paper (Whatman, Inc., Clifton, N.J.) and developed by the ascending chromatography technique in a solvent system of *n*-butanol–pyridine–water (6:4:3) at room temperature. Sugars were detected by the dipping method with silver nitrate-sodium hydroxide reagent (12).

High-performance liquid chromatography. Sugar products were finally identified by high-performance liquid chromatography (model TRI ROTER-V; JASCO Co. Ltd., Japan) equipped with a packed column (4.6 by 250 mm; PA-03; YMC) and a refractive index detector (RID-6A; Shimadzu Co., Ltd., Japan). Ten microliters of the reaction mixture was injected onto the column. The elution of sugars was performed with 60% acetonitrile.

Scanning electron microscopic observation of digested starch granules. For scanning electron microscopy, starch granules recovered from the reaction mixture were dried at the critical point in a critical point dryer (JCPD-5; Japan Electron Opticus Laboratories, Ltd., Japan), coated with gold with an enscope sputter coater (JEOL Ltd.) and observed with a scanning electron microscope (JSM 25S; JEOL Ltd.).

RESULTS

Selection of a raw-starch-digesting α-amylase-producing bacterium. About 800 isolates were plated individually onto raw-corn-starch-mineral-agar medium and were cultivated in nutrient agar medium for 3 to 4 days. The isolates were plated individually onto raw-corn-starch-mineral-agar medium and cultivated. First, 15 colonies on the medium with larger halos (clear zones) were selected, and several transfers were repeated on nutrient broth slants. Then, the colonies were plated onto raw-corn-starch-mineral-agar medium and a second set of four strains was selected. These isolates were tested further for their ability to produce α-amylase in nutrient broth medium on a shaking culture; and finally, strain 65, which produced the largest amount of α-amyase, was selected.

Characterization of the isolated strain. Physiological and biochemical tests were carried out on strain 65 by the methods described in *Bergey's Manual of Determinative Bacteriology* (1). Strain 65 was aerobic, rod shaped (width, 0.6 to 0.8 μm; length, 1.8 to 0.8 μm), gram positive, motile, and spore forming. It grew at 30 to 50°C at a pH range of 6.0 to 10.0 and an NaCl concentration of 0 to 5%; but it could not grow at 4°C, pH 3.5, or an NaCl concentration of 10.0%. Acid was produced from G1, arabinose, xylose, and mannitol; but gas was not produced. Citrate was assimilated. It was positive by the Voges-Proskauer test and was negative by the methyl red test. Catalase was produced, casein was hydrolyzed, and gelatin was liquefied, but diffusible pigment was not formed. According to these data, this strain was identified as *B. subtilis* 65.

Enzyme production. *B. subtilis* 65 was cultivated at 30°C in 120 liters of nutrient broth in a 200-liter stainless steel fermentor; the pH was not controlled. Maximum α-amylase activity (68 mU) was obtained after 24 h.

Purification of α-amylase. The lyophilized preparation of culture filtrate was dissolved in 0.05 M Tris hydrochloride buffer (pH 8.0) and applied to a Sephacryl S-300 column. Fractions of α-amylase activity were combined and applied to a CM Sephadex C-50 column. The α-amylase fractions were combined and lyophilized. The recovery and the specific activity of the enzyme are given in Table 1. The purified preparation was homogeneous, as determined by disc gel electrophoresis (Fig. 1).

Properties of α-amylase. (i) Molecular weight and molecular parameters. The molecular weight of the α-amylase from strain 65 was estimated by sodium dodecyl sulfate-polyacryl-

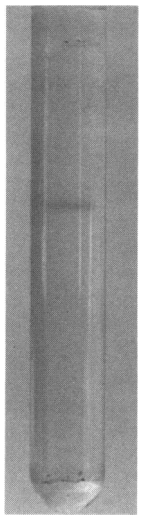


FIG. 1. Disc gel electrophoretic pattern of purified α-amylase from *B. subtilis* 65.

amide gel electrophoresis to be 68,000. The sedimentation coefficient (s_{20} value) and the Stokes radius of this enzyme molecule were 4.28 and 35 Å, respectively.

(ii) Optimal temperature and pH. The enzyme exhibited maximum activity at 60°C (Fig. 2A) and pH 6.0 (Fig. 2B).

(iii) Thermal and pH stability. The α-amylase from strain 65 was rather unstable at higher temperatures and was unstable when it was kept at more than 60°C for 5 min (Fig. 3A). To determine the pH stability, *B. subtilis* 65 α-amylase was dissolved in the following buffer systems: Clark-Lubs (pH 0.1 to 2.0), Sørensen citrate (pH 2.5 to 5.0), Sørensen phosphate (pH 5.5 to 7.5), and Clark borate (pH 8.0 to 10.0). After the mixture was kept at 4°C for 24 h, the residual activity was measured under standard assay conditions. This α-amylase was stable at pH 6.0 to 9.0 (Fig. 3B).

TABLE 1. Purification of raw-starch-digesting α-amylase from *B. subtilis* 65

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Recovery (%)
Crude filtrate	4,000	6,684	2,304	0.35	100
20 to 50% $(NH_4)_2SO_4$ fractionation	210	589.5	2,277	3.86	97.3
Sephacryl S-300	107	171.8	783	4.56	33.5
CM Sephadex C-50	90	57.3	581	10.8	24.8

(iv) Effect of metal ions and chemical reagents. Cu^{2+}, Fe^{3+}, Mn^{2+}, and Hg^{2+} inhibited the enzyme activity almost completely at concentrations of 2 mM. Zn^{2+}, Pb^{2+}, Al^{3+}, Cd^{2+}, and Ag^{+} inhibited the enzyme activity by 30.3, 38.0, 38.7, 43.0, and 61.2%, respectively. Ba^{2+}, Fe^{2+}, Li^{+}, Cs^{2+}, Co^{2+}, Sr^{2+}, Mg^{2+}, Ni^{2+}, and Ca^{2+} did not inhibit the enzyme, nor did iodoacetamide (0.1 mM) or *p*-chloromercuribenzoate (0.1 mM). EDTA (0.1 mM) inhibited the enzyme activity by 61.3%. Moreover, when the enzyme was dialyzed without Ca^{2+}, it was completely inactivated and did not recover its activity on redialysis against 10 mM $CaCl_2$ (data not shown). These results indicate that this α-amylase requires Ca^{2+} for its stabilization.

(v) Digestion of glycogen and gelatinized potato starch. Hydrolysis curves for the α-amylase from strain 65 and the liquefying α-amylase for glycogen and gelatinized potato starch were compared. The digestion limits of glycogen and gelatinized potato starch were 40 and 60%, respectively, which were the same as those for liquefying α-amylase and fungal α-amylase I.

(vi) Digestion of various raw starches. The hydrolysis curves exhibited by the α-amylase from strain 65 for various samples of raw starch are shown in Fig. 4. This enzyme digested waxy corn starch, corn starch, sweet potato starch, and wheat starch by 39, 36, 35, and 32% as glucose, respectively, at pH 6.0 and 30°C. It characteristically di-

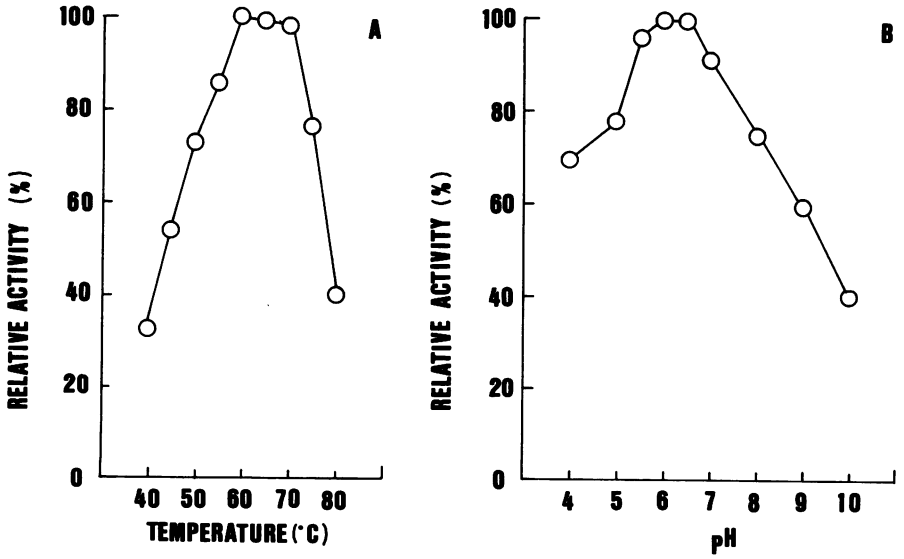


FIG. 2. Optimal temperature (A) and pH (B) for the maximum activity of α-amylase from strain 65. (A) Effects of temperature on activity were determined. The reaction mixture contained 5 ml of gelatinized potato starch, 1 ml of pure water, 1 ml of 0.1 M acetate buffer (pH 6.0), and 1 ml of enzyme. α-Amylase from strain 65 was incubated at various temperatures, and the relative activities were measured. (B) Effects of pH on enzyme activity were determined at 60°C by using the following buffer systems: Clark-Lubs (pH 0.1 to 2.0), Sørensen citrate (pH 2.5 to 5.0), Sørensen phosphate (pH 5.5 to 7.5), and Clark borate (pH 8.0 to 10.0). Experimental details are given in the text.

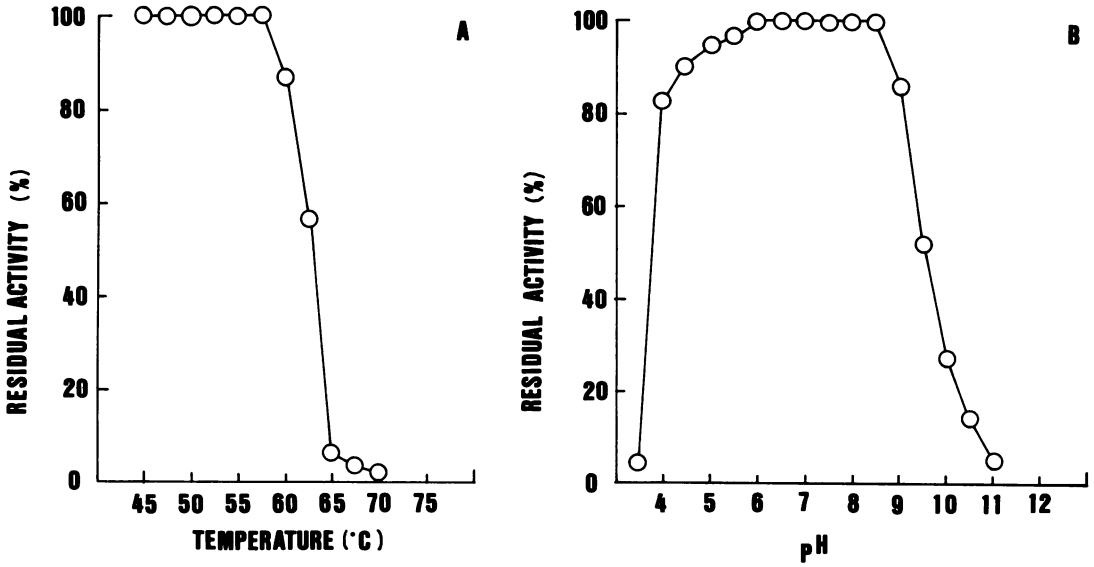


FIG. 3. Thermal stability (A) and pH stability (B) of α-amylase from strain 65. For thermal stability, each enzyme solution was kept at various temperatures for 5 min, and the residual activity was measured. For pH stability, the enzyme was dissolved in buffers with various pHs and was kept at 4°C for 24 h. Then, the residual activity was measured. Experimental details are given in the text.

gested potato starch by 30% as glucose. The sugar products in the reaction mixture were G1, G2, G3, G4, G5, and larger maltooligosaccharides by paper chromatography (data not shown). Sugar products from various raw starches were determined to be G1, G2, G3, G4, G5, G6, and G7 by high-performance liquid chromatography. The molar ratios of maltooligosaccharides from raw corn starch after 5 days of reaction were 1.00, 1.52, 0.90, 0.13, 0.07, 0.02, and 0.01, respectively (Fig. 5).

(vii) Raw starch adsorbability. The raw-starch-digesting α-amylase from strain 65 showed no adsorbability onto raw corn starch at any pH.

(viii) Digestion of PNP-G1 and PNP-G2. The actions of both bacterial α-amylases, raw-starch-digesting and liquefying, on PNP-G1 and PNP-G2 were compared. Raw-starch-digesting α-amylase from *B. subtilis* 65 digested PNP-G2 to G2 and *p*-nitrophenol, but it could not digest G2 or PNP-G1. A total of 16.0 μmol of *p*-nitrophenol was liberated. Therefore, PNP-G2 was completely hydrolyzed in 8 h. On the contrary, liquefying α-amylase did not liberate *p*-nitrophenol from PNP-G2, but 0.12 mg of the reducing sugar per ml was produced. Neither PNP-G1 nor G2 was hydrolyzed by this enzyme. These data indicate that the hydrolysis of PNP-G2 by raw-starch-digesting α-amylase occurs at the linkage between *p*-nitrophenyl and G2, and liquefying α-amylase hydrolyzes the linkage between two G1 residues.

(ix) Action pattern of α-amylases. The action pattern of α-amylases toward the maltooligosaccharides was determined by high-performance liquid chromatography. α-Amylase from *B. subtilis* 65 digested G4, G5, and G6 to G1, G2, and G3, respectively, and digested G3 to G1 and G2; but even after 48 h of reaction, residual G3 was present. The final molar ratio of G1, G2, and G3 was 0.309, 0.424, and 0.559, respectively. However, liquefying α-amylase showed weaker digestibility to oligosaccharides. G3 in particular could not be digested by this enzyme at all (Fig. 6).

The relative velocities of *B. subtilis* 65 α-amylase to soluble starch, amylose, G5, and G3 were 100, 28, 8, and 3, respectively. The K_m values for amylose, G5, and G3 were 3.50×10^{-6}, 1.20×10^{-4}, and 1.98×10^{-4} M, respectively.

(x) Scanning electron microscopic observation of digested starch granules. The fine structure of the digested starch granules was observed by scanning electron microscopy. Starting points of raw corn starch digestion of fungal α-amylase I were 3 to 5 for each starch granule. On the contrary, the starting holes of raw corn starch digestion of fungal glucoamylase I looked like pin holes. In the case of *B. subtilis* 65 α-amylase, the starting holes of raw corn starch

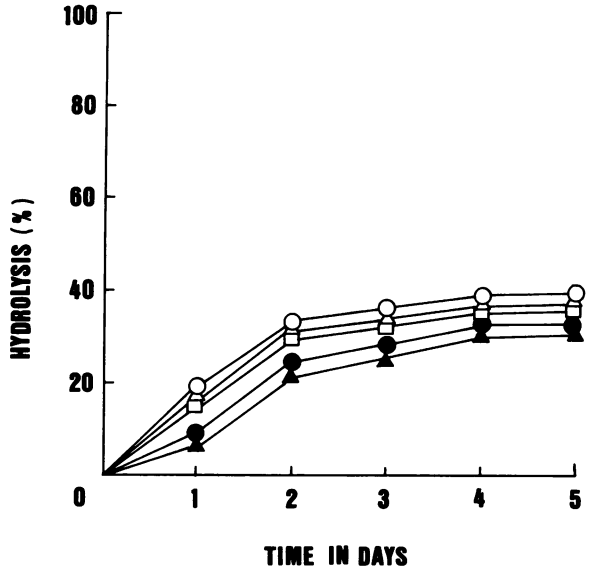


FIG. 4. Digestion of various raw starches by *B. subtilis* 65 α-amylase. Symbols: ○, digestion curve of *B. subtilis* 65 α-amylase with waxy corn starch; △, with corn starch; □, with sweet potato starch; ●, with wheat starch; ▲, with potato starch. The reaction mixture, which contained 50 mg of raw corn starch, 4 ml of 0.1 M acetate buffer (pH 6.0), 4 ml of enzyme solution (2 U/ml), and 1 drop of chloromycetin solution (50 μg/ml of ethanol), was incubated at 30°C.

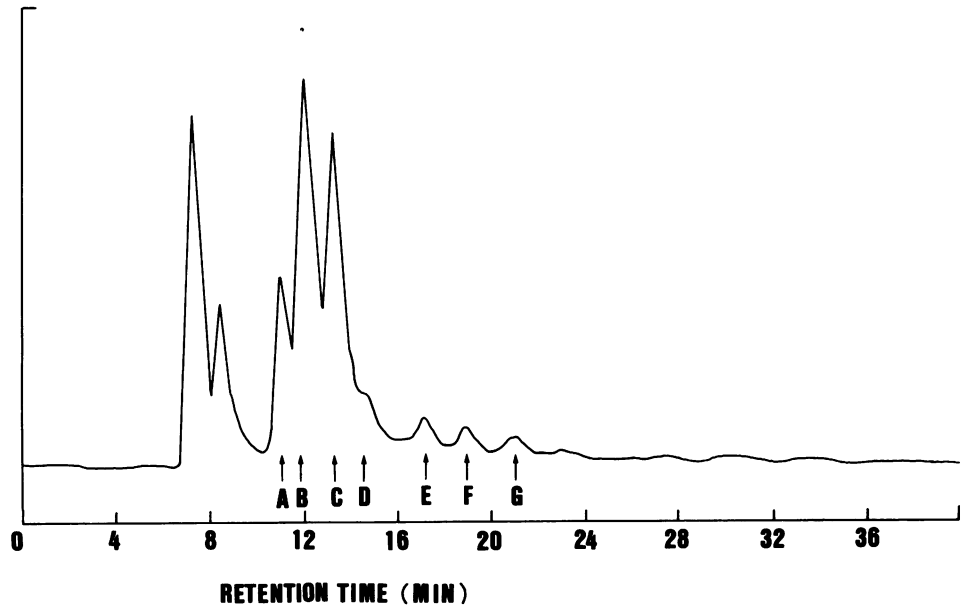
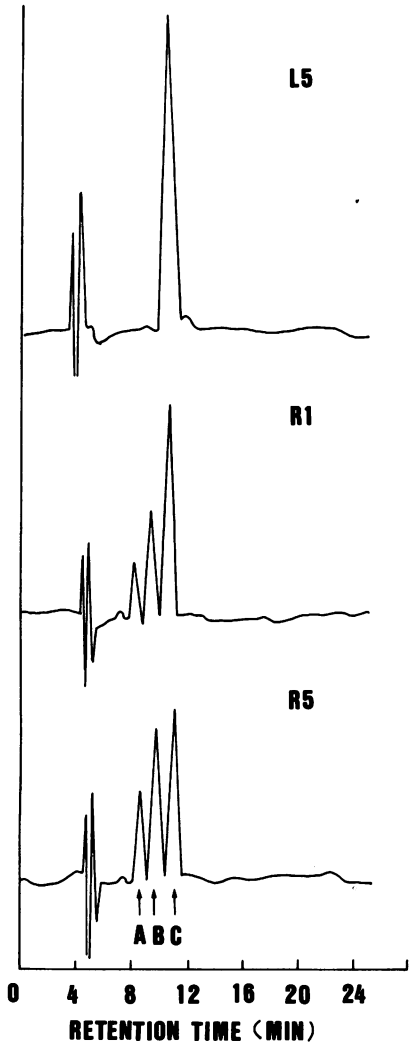


FIG. 5. Sugar products from raw corn starch. Raw corn starch (50 mg) was mixed with 8 ml of α-amylase solution (2.0 U/ml) and incubated at 30°C for 5 days. Protein was removed by ultrafiltration, and the reaction mixture was applied to a high-performance liquid chromatograph. Elution was performed with 60% acetonitrile at a flow rate of 0.5 ml/min. A, G1; B, G2; C, G3; D, G4; E, G5; F, G6; G, G7.



digestion were similar to those of glucoamylase I (Fig. 7A). The holes and surfaces of digested granules had no regularity, and then the holes gradually widened; but the size, depth, and width were different for each hole. *B. subtilis* 65 α-amylase also digested potato starch granules (Fig. 7B). The holes were widened, and digestion progressed as shown in Fig. 7C; and potato starch granules were digested and solubilized to sugars, as shown in Fig. 7D.

(xi) Synergistic effect with glucoamylase in the digestion of raw potato starches. The raw-starch-digesting α-amylase from *B. subtilis* 65 exhibited a high synergistic effect with the glucoamylase from *Endomycopsis fibligera* in the digestion of raw potato starches. Raw potato starch was completely digested to G1, as shown in Fig. 8.

DISCUSSION

We tried to isolate bacteria which could produce large amounts of the raw-starch-digesting α-amylase and certified the presence of a novel type of raw-potato-starch-digesting α-amylase.

The purified preparation of this α-amylase was homogeneous on disc gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and it hydrolyzed gelatinized potato starch and glycogen by 60 and 40%, respectively. The molecular weight of the α-amylase was estimated to be 68,000 by sodium dodecyl sulfate-polyacryl-

FIG. 6. Action pattern of α-amylases to the maltooligosaccharides. A total of 3 ml of 0.5% maltooligosaccharide solution was mixed with 0.3 ml of α-amylase solution (1.0 U/ml) and incubated at 30°C. Protein was removed by ultrafiltration and applied to a high-performance liquid chromatograph. Elution was performed with 60% acetonitrile at a flow rate of 0.4 ml/min. A, G1; B, G2; C, G3. L5, G3 was incubated with liquefying α-amylase for 5 min; R1, G3 was incubated with *B. subtilis* 65 α-amylase for 1 min; R5, G3 was incubated with *B. subtilis* 65 α-amylase for 5 min.

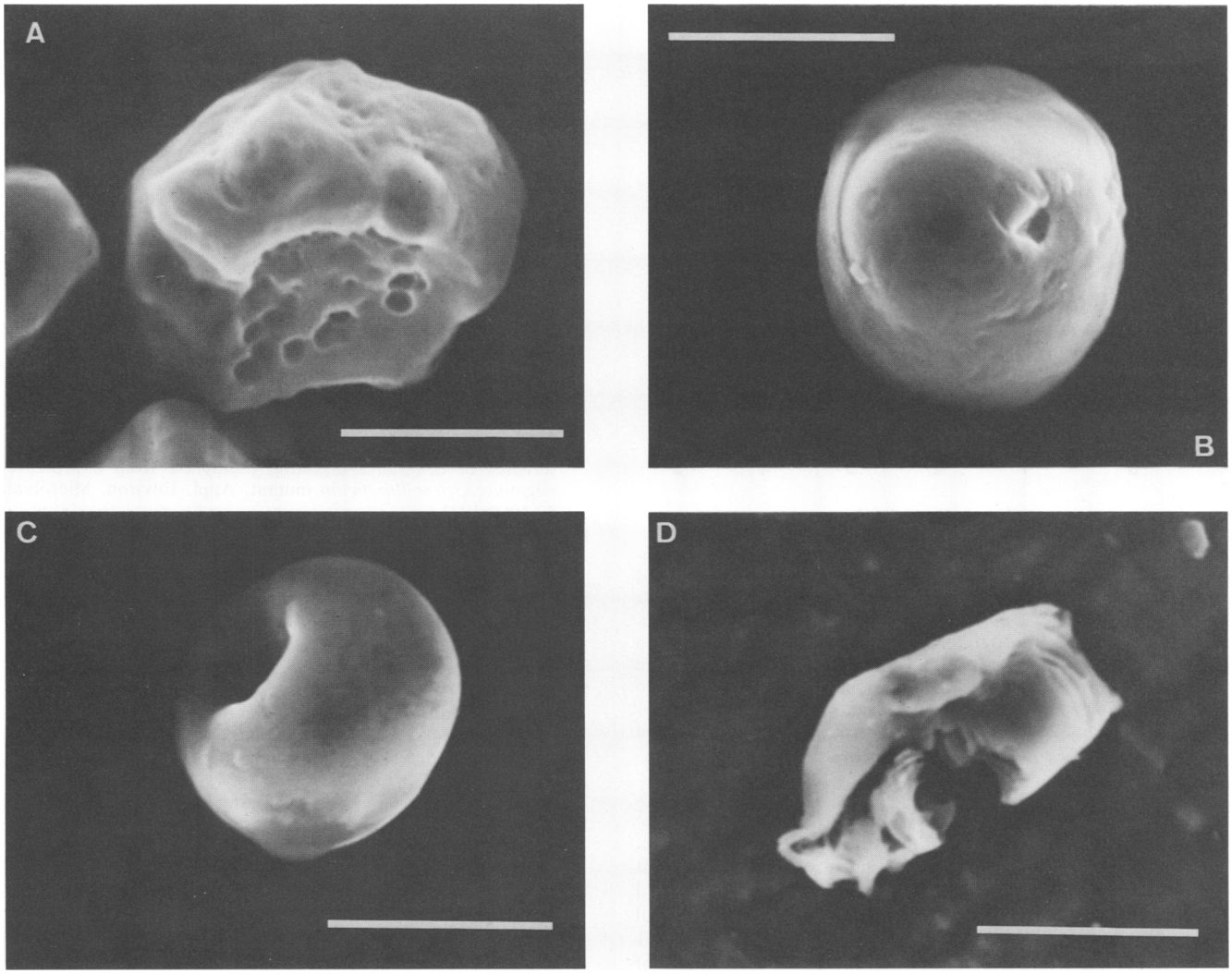


FIG. 7. Scanning electron micrograph of digested corn starch granule and potato starch granule. The corn starch granule was incubated with *B. subtilis* 65 α-amylase for 2 days. (A) The potato starch granule was incubated with *B. subtilis* 65 α-amylase for 2 days (B), 3 days (C), and 4 days (D). Digested starch granules were collected and dried in a critical point dryer (JEOL Ltd.) and used as the sample for scanning electron microscopy. Bars, 10 μm.

amide gel electrophoresis. *B. subtilis* 65 α-amylases produced G1, G2, G3, G4, G5, G6, and G7 from all kinds of raw starches.

In fungal α-amylases and glucoamylases, the occurrence of the raw starch digestibility was parallel with the raw starch adsorbability, which occurred at the raw starch affinity site, which is different from the active site on the enzyme molecule (2, 4, 7). This raw-starch-digesting α-amylase showed no adsorbability onto raw corn starch at any pH. This is different from that observed with the raw starch-digesting *A. ficum* α-amylase I (8), which showed raw starch adsorbability, as described previously. *B. subtilis* 65 α-amylase digested potato starch granules, although the fungal amylases hardly digested potato starch granules, and it exhibited a high synergistic effect with the glucoamylase from *E. fibligera* on potato starch digestion. The mixed preparation of these enzymes would be available for the hydrolysis of raw potato starch. On the contrary, bacterial liquefying α-amylase was not able to adsorb or digest raw starch.

Scanning electron microscopic observations revealed that the digestion pattern of *B. subtilis* 65 α-amylase to the raw corn starch resembled that of glucoamylase I. There were many holes on the surface of the digested starch granule, but the sizes, depths, and widths of the holes were different. In the case of fungal α-amylase I and glucoamylase I, the digested holes were similar in size during the same digestion period. This α-amylase had no raw starch adsorbability, and the raw starch digestion depended on the thrust between the enzyme and the starch granule. Because of this thrust, digested starch showed random style. In the case of fungal amylases, however, raw starch digestion was closely related to raw starch adsorption and the digested starch had regularity.

Only one raw-starch-digesting α-amylase of *Bacillus circulans* has been shown (6, 10) to produce G6 from the gelatinized potato starch in the early stages of hydrolysis, and then the G6 was hydrolyzed to G4 and G2.

The actions of strain 65 α-amylase and liquefying α-amylase on PNP-G1 and PNP-G2 were compared. Neither of

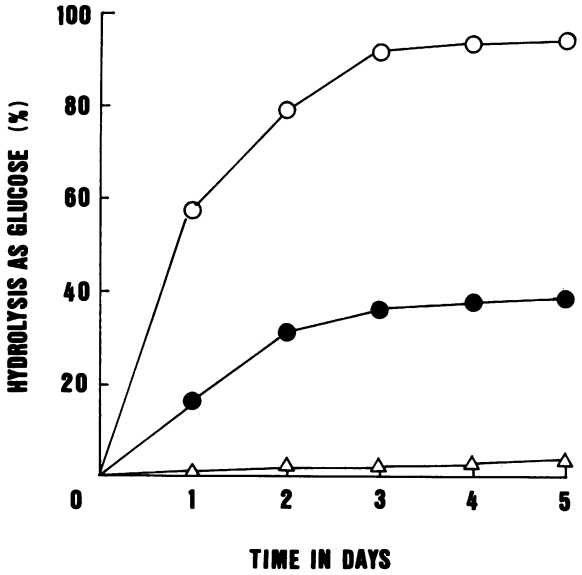


FIG. 8. Synergistic effect of *B. subtilis* 65 α-amylase and *Endomycopsis* glucoamylase. Symbols: ○, digestion curve of a mixed enzyme preparation of *B. subtilis* α-amylase and *Endomycopsis* glucoamylase; ●, *B. subtilis* 65 α-amylase; △, *Endomycopsis* glucoamylase. The reaction mixture contained 50 mg of raw potato starch, 4 ml of enzyme solution (2.0 U/ml), and 4 ml of 0.1 M acetate buffer (pH 6.0). For synergistic digestion, 50 mg of raw potato starch, 3 ml of *B. subtilis* 65 α-amylase, 3 ml of *Endomycopsis* glucoamylase, and 2 ml of acetate buffer were mixed and incubated at 30°C.

the enzymes could digest PNP-G1 or G2. *B. subtilis* 65 α-amylase showed a strong digestibility toward PNP-G2. This enzyme digested PNP-G2 to G2 and *p*-nitrophenol. But liquefying α-amylase showed a weaker digestibility toward PNP-G2. Different from *B. subtilis* α-amylase, *p*-nitrophenol was not liberated, but trace amounts of glucose and PNP-G1 were liberated from the reaction mixture. The actions of these two enzymes toward the small substrate were different.

We therefore expect that the raw starch digestion mechanism of *B. subtilis* α-amylase is different from that of fungal α-amylase I and glucoamylase I. We will try to clarify the mechanism of raw starch digestion by gene technology.

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