Production and Characteristics of Raw-Starch-Digesting α-Amylase from a Protease-Negative *Aspergillus ficum* Mutant

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Mutational experiments were carried out to decrease the protease productivity of *Aspergillus ficum* IFO 4320 by using N-methyl-N'-nitro-N-nitrosoguanidine. A protease-negative mutant, M-33, exhibited higher α-amylaseactivity than the parent strain under submerged culture at 30°C for 24 h. About 70% of the total α-amylase activity in the M-33 culture filtrate was adsorbed onto starch granules. The electrophoretically homogeneous preparation of raw-starch-adsorbable α-amylase (molecular weight, 88,000), acid stable at pH 2, showed intensive raw-starch-digesting activity, dissolving corn starch granules completely. The preparation also exhibited a high synergistic effect with glucoamylase I. A mutant, M-72, with higher protease activity produced a raw cornstarch-unadsorbable α-amylase. The purified enzyme (molecular weight, 54,000), acid unstable, showed no digesting activity on raw corn starch and a lower synergistic effect with glucoamylase I in the hydrolysis of raw corn starch. The fungal α-amylase was therefore divided into two types, a novel type of raw-starch-digesting enzyme and a conventional type of raw-starch-nondigesting enzyme.

Many reports have been published on fungal α-amylase (α-1,4-D-glucan 4-glucanohydrolase; EC 3.2.1.1 [endo-amylase]), but no reports could be found on a raw-starch-digesting fungal α-amylase.

As reported previously (4), *Aspergillus awamori* var. *kawachi* produced three kinds of glucoamylase: raw-starch-digesting glucoamylase GA I (molecular weight [MW] 90,000; type A) and raw-starch-nondigesting glucoamylase GA I' (MW 83,000; type B) and GA II (MW 57,000; type C). The multiplicity of the enzyme was ascribed to the stepwise degradation of the original glucoamylase GA I by protease and glucosidases (17). The occurrence of raw starch digestibility was paralleled by raw starch adsorbability (7), which occurred at the raw starch affinity site, different from the active site in the GA I molecule. This raw starch affinity site was specifically cleaved by protease, leading to the formation of raw-starch-nondigesting GA I'. We further showed that the protease-negative, glycosidase-negative mutant strain HF-15 of *A. awamori* var. *kawachi* produced only raw-starch-digesting glucoamylase GA O (MW 250,000; type A) under any conditions of submerged or solid culture (3, 5, 6). On the basis of these results with glucoamylase, we expected the presence of raw-starch-digesting α-amylase from fungi and tried to isolate the protease-negative mutant that could produce a high amount of a raw-starch-adsorbable, raw-starch-digesting α-amylase.

This paper describes the isolation of a protease-negative mutant from *A. ficum* IFO 4320 and the production, purification, and properties of raw-starch-digesting α-amylase. For comparison, a raw-starch-nondigesting α-amylase from a hyper-protease mutant of the same parent strain is also described.

MATERIALS AND METHODS

Induction and isolation of mutants. *A. ficum* IFO 4320 was used as parent material. The parent culture was grown on a

slant of complete medium (glucose, 10 g; peptone, 5 g; yeast extract, 5 g; malt extract, 5 g; KH₂PO₄, 1 g; agar, 20 g; deionized water, 1,000 ml) for 10 days at 30°C. The spores were collected and suspended in sterile water to give a spore suspension not less than 10^8 spores per ml. A 10-ml portion of spore suspension was treated with 10 ml of freshly prepared N-methyl-N'-nitro-N-nitrosoguanidine at a concentration of 4 mg/ml in 0.1 M acetate buffer, pH 4.5, for 20 min in a sterilized tube and then centrifuged for 5 min to remove the supernatant. The centrifuged spores were washed twice with sterile water and then inoculated into 100 ml of minimal medium (glucose, 50 g; NaNO₃, 3 g; KH₂PO₄, 1 g; KCl, 0.5 g; MgSO₄ · 7H₂O, 0.2 g, FeSO₄ · 7H₂O, 0.01 g; deionized water, 1,000 ml) and shaken at 30°C for 36 h. The mycelia were separated by filtration through sterile glass wool, and the filtrate was plated onto complete medium slants and further incubated at 30°C for 7 to 10 days. Several transfers were done to test the stability of the mutants. The selected mutants were plated individually onto casein medium for primary screening for protease productivity. Mutants with significantly large and small halos (clear zones) compared with the parent were selected and cultured in potato dextrose agar medium for 10 days at 30°C. Spores of these selected mutants were further suspended in sterile pure water to give a spore suspension of not less than 10^8 spores per ml. A 10-ml amount from each suspension was treated with 10 ml of freshly prepared N-methyl-N'-nitro-N-nitrosoguanidine (4 mg/ml) in 0.1 M acetate buffer, pH 4.5, for 20 min in sterile tubes and then centrifuged for 5 min to remove the supernatant. The centrifuged spores were washed once with sterile water, plated onto complete medium, and further incubated at 30°C for 7 days. The stable mutants were plated onto complete medium to assess their stability. The isolates were tested for their ability to produce α-amylase which had adsorbability to raw corn starch in liquid synthetic medium A (potato starch, 40 g; ammonium citrate, 10 g; KH₂PO₄, 3 g; CaCl₂, 0.1 g; Fe₂(SO₄)₃ · 7H₂O, 0.01 g; MgSO₄ · 7H₂O, 1 g; deionized water, 1,000 ml).
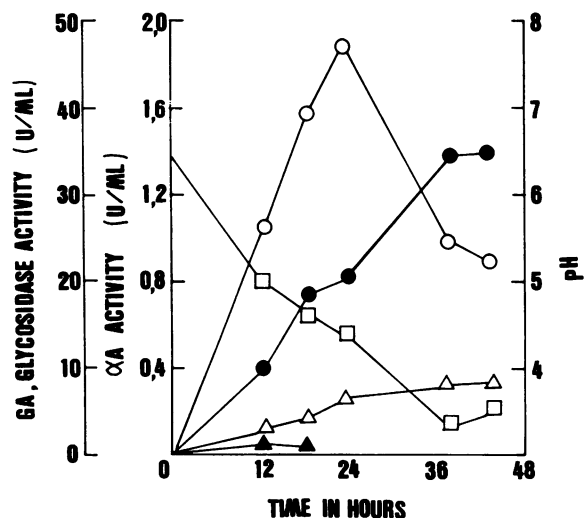
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FIG. 1. Time course of enzyme production of α-amylase from mutant M-33 under submerged culture condition. Symbols: ●, glucoamylase activity; ○, α-amylase activity; □, pH; △, N-acetyl-β-D-glucosaminidase activity; ▲, α-mannosidase activity. Experimental details are described in the text.

Culture of mutants. Selected mutants were grown on potato dextrose agar slants for 10 days. The spores were collected, inoculated into 100 ml of liquid synthetic medium A, and cultured with shaking for 2 days at 30°C. The culture filtrate was used for enzyme assay.

Enzyme production. For seed cultures, portions (1 liter) of synthetic medium A were dispensed into 5-liter round flat-bottomed flasks, autoclaved at 121°C for 20 min, and inoculated with spores from slant cultures of mutants. Incubation of the inoculated medium was carried out on a reciprocating shaker at 30°C for 2 days. For the main submerged culture, 120 liters of synthetic medium A was sterilized at 121°C for 20 min in a 200-liter stainless-steel fermentor (Marubishi Bioeng. Co. Ltd.), inoculated with agitation at 200 to 250 rpm, and aerated at a rate of 1 volume of air per volume of medium per min.

Assay of glycosidase activity. p-Nitrophenyl-α-D-mannoside and p-nitrophenyl-N-acetyl-β-D-glucosaminide, both

TABLE 1. Purification of raw-starch-adsorbable α-amylase of A. ficum mutant M-33

Purification step	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg of protein)	Recovery (%)
Crude filtrate	4,000	112 × 10²	5,110	2.18	100
60% (NH₄)₂SO₄ fractionation	400	18.1 × 10²	445	4.07	16.2
After acetone treatment	400	9.05 × 10²	111	8.15	8.1
20 to 50% (NH₄)SO₄ fractionation	105	7.57 × 10²	83	9.12	6.8
Sephacryl S-300	205	7.06 × 10²	66	10.7	6.3
DEAE-Cellulofine AH	120	6.76 × 10²	41	16.5	6.1

purchased from Sigma Chemical Co., were used as substrates for the assay of α-mannosidase and N-acetyl-β-D-glucosaminidase, respectively. The incubation mixture contained 0.1 ml of enzyme activity and 0.9 ml of 0.01% substrate in citrate buffer, pH 4.3. The mixtures were incubated at 37°C for 30 min and then 2 ml of 0.25 M sodium carbonate was added to the mixture. The p-nitrophenol liberated was determined spectrometrically at 400 nm. One unit of enzyme activity was defined as the amount of enzyme
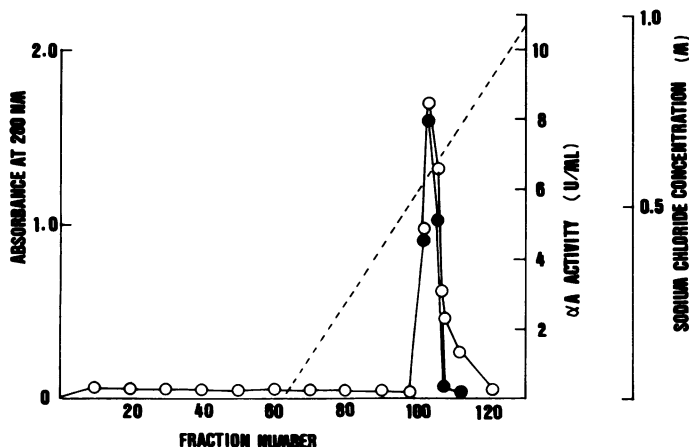

FIG. 2. DEAE-Cellulofine AH chromatography of raw-starch-adsorbable α-amylase from mutant M-33. Symbols: ○, A₂₈₀; ●, α-amylase activity. Experimental details are described in the text.

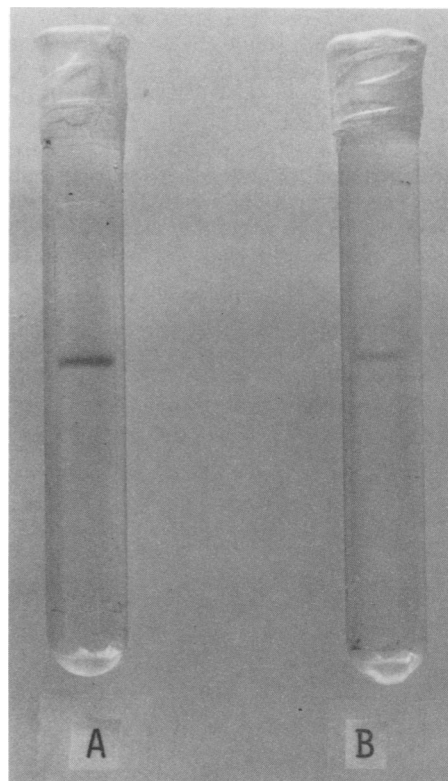FIG. 3. Disc electrophoretic pattern of purified α-amylase from mutants M-33 (A) and M-72 (B). Polyacryl (7.5%) gel column, pH 7.3. About 10 μg of α-amylase was used, and 2 mA per column (0.5 by 8 cm) was applied for 120 min. Staining was done with 0.005% Coomassie brilliant blue R-250.
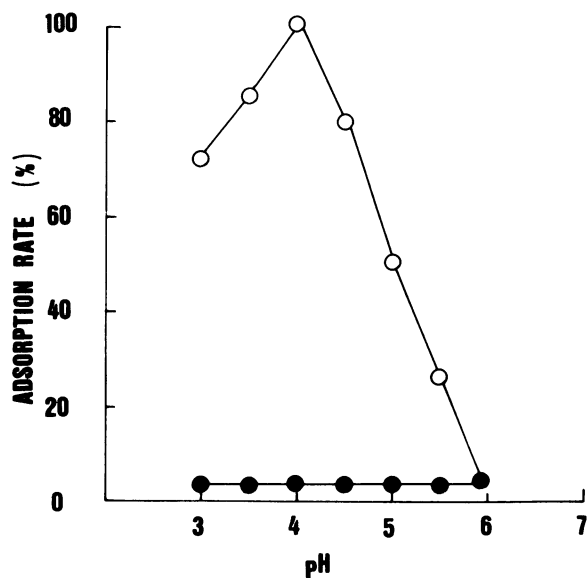
FIG. 4. Effect of pH on adsorption of α-amylase onto raw corn starch granule. Symbols: O, M-33 α-amylase; ●, M-72 α-amylase. Experimental details are described in the text.

that liberated 1 μm of p-nitrophenol per min under the above conditions.

Assay of amylase activity. Glucoamylase activity was determined according to the methods described previously (4). α-amylase activity was determined by incubating 1 ml of appropriately diluted enzyme solution with 5 ml of 1% gelatinized potato starch solution (Japanese Pharmacopeia), 1 ml of 0.1 M acetate buffer (pH 6.0), and 1 ml of pure water at 45°C. After 10 min, reducing sugar formed was determined by the micro-Bertrand method (1, 10). One unit of α-amylase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar from the substrate per min.

Adsorption rate of α-amylase onto raw corn starch. The desired amount of culture filtrate was added to 0.02 M acetate buffer, pH 4.0, to prepare an α-amylase solution of
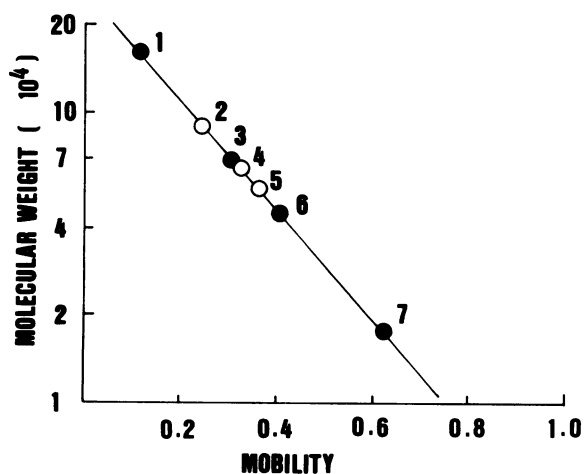

FIG. 5. Estimation of MW by sodium dodecyl sulfate electrophoresis. MWs of standard proteins are given in parentheses: 1, gamma globulin (160,000); 2, A. ficum M-33 α-amylase (88,000); 3, bovine albumin (67,000); 4, A. ficum IFO 4320 α-amylase (64,000); 5, A. ficum M-72 α-amylase (54,000); 6, ovalbumin (45,000); 7, myoglobin (17,800).

2.0 U. One gram of raw corn starch was added to 5 ml of the prepared solution and left to stand at 4°C for 15 min. After centrifugation α-amylase activity of the supernatant fluid was assayed and compared with that of the original α-amylase solution. The adsorption rate (AR) is defined according to the equation $AR(\%) = [(B - A)/B] \times 100$.

Purification of two types of α-amylase. Based on the procedure above, mutants M-33 and M-72 were cultivated, and the resulting culture filtrates were collected for enzyme purifications.

(i) Step 1. Ammonium sulfate was added to an extent of 60% concentration to the culture filtrate and kept overnight at 4°C. The resulting precipitate was collected by filtration with Celite (Johns-Manville Products Corp.). Crude amylase, 120 g, was suspended in 4,000 ml of cold 0.125 M calcium acetate solution, and the solution was adjusted at pH 7.0 by gradual addition of 5.0 N sodium hydroxide. The solution was stirred with a magnetic stirrer for 3 h at 4°C, and then insoluble material was removed by centrifugation (6,000 rpm, 15 min). Solid ammonium sulfate was added to a 50% concentration. The solution was left to stand overnight at 4°C. The precipitate was collected by centrifugation (8,000 rpm, 20 min) and washed twice with 60% ammonium sulfate. The precipitate was dissolved in a small amount of 0.1 M acetate buffer (pH 6.0) containing 0.002 M calcium chloride and was dialyzed against the same buffer. Insoluble material was removed by centrifugation. Acetone was added dropwise to the enzyme solution to a 55% concentration. The precipitate was collected by centrifugation (8,000 rpm, 15 min). The precipitate was dissolved in a minimal amount of 0.02 M calcium acetate solution, and again cold acetone was carefully added to the solution until it became slightly turbid. On storage of this preparation in a cold room, precipitate was obtained within 1 day.

(ii) Step 2. Chromatography on Sephacryl S-300 was performed. The enzyme precipitate as above was dissolved in a small volume of 0.1 M acetate buffer (pH 6.0) and applied to a Sephacryl S-300 column (2.5 by 100 cm) previously equilibrated with 0.1 M acetate buffer (pH 6.0). Filtration was carried out with the same buffer at 4°C at a rate of 10 ml/25 min. α-Amylase activity was recovered in fractions 72 to 85 (each fraction, 4 ml).

(iii) Step 3. The α-amylase obtained by Sephacryl S-300 chromatography was placed on a DEAE-Cellulofine AH column (2.3 by 57 cm) previously equilibrated with 0.1 M acetate buffer (pH 6.0). The column was washed with several column volumes of buffer, and the α-amylase was eluted with a linear gradient of 0 to 0.7 M sodium chloride in 0.1 M acetate buffer (pH 6.0). α-Amylase activity was recovered in fractions 99 to 106 (each fraction, 4 ml).

Determination of raw starch digestion. A reaction mixture containing 0.3 g of raw corn starch, 36 ml of pure water, 6 ml of α-amylase solution (2.0 U/ml), 6 ml of 0.1 M acetate buffer, (pH 5.2), and 1 ml of toluene was incubated at 30°C. At suitable intervals, reducing sugar formed in 1 ml of the reaction mixture was determined by the micro-Bertrand method and the degree of hydrolysis was calculated (1).

Scanning electron microscopic observation of starch granules. For scanning electron microscopy, starch granules recovered from the reaction mixture were dried at the critical point in a JCPD-5 critical-point dryer (Japan Electron Optics Laboratory [JEOL] Ltd.), coated with gold with an Enscope sputter coater (JEOL Ltd.), and observed with a scanning electron microscope, JSM 25S (JEOL Ltd.) (13, 14).

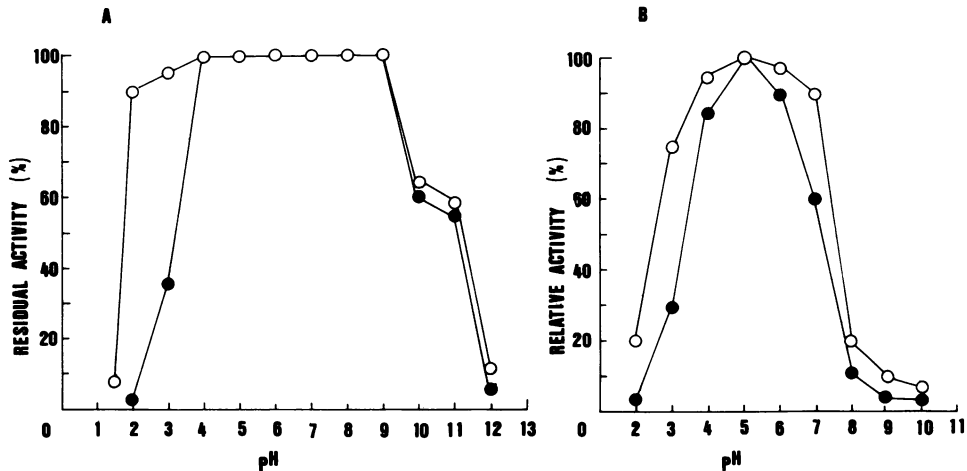**General analytical procedures.** Disc gel electrophoresis in

FIG. 6. pH stability (A) and optimal pH (B) of M-33 and M-72 α-amylases. To determine the pH stability, both enzymes were 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); Clark borate (pH 8.0 to 10.0). The mixtures were kept at 4°C for 24 h. The residual activity was measured under standard assay conditions.

7.5% polyacrylamide gel at pH 7.3 in Tris-asparagine buffer was carried out by the method of Hendrick and Smith (9). Molecular weights were estimated by sodium dodecyl sulfate electrophoresis, using a 5.0% polyacrylamide gel (15).

RESULTS

Selection of fungal strains producing raw-starch-adsorbable α-amylase. More than 300 strains of black aspergilli and other fungal strains were isolated and cultured in solid wheat bran medium. The extracts were assayed for raw starch adsorbability of α-amylase. *A. ficum* IFO 4320 was selected as the best producer.

Selection of mutants. At first 10 colonies with larger and smaller halos on casein medium were primarily selected in about 300 colonies of *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine-treated parent strains and were transferred to potato dextrose agar slant. Several transfers were repeated on the slant and then plated onto casein medium. Thirty strains were secondarily selected on the basis of higher and lower ratios of halo diameter/colony diameter on the casein medium. These isolates were further tested for their ability

to produce protease and α-amylase in solid wheat bran medium. Mutant M-33, exhibiting the lowest protease activity, showed the highest adsorbability of α-amylase activity onto corn starch granules. Mutant M-72, which increased in protease productivity and showed no adsorbability of α-amylase activity onto corn starch granules, was selected.

Enzyme production. Mutants M-33 and M-72 were cultivated at 30°C in 120 liters of synthetic medium A in a 200-liter stainless-steel fermentor without pH control. Time courses of enzyme production are shown in Fig. 1. The
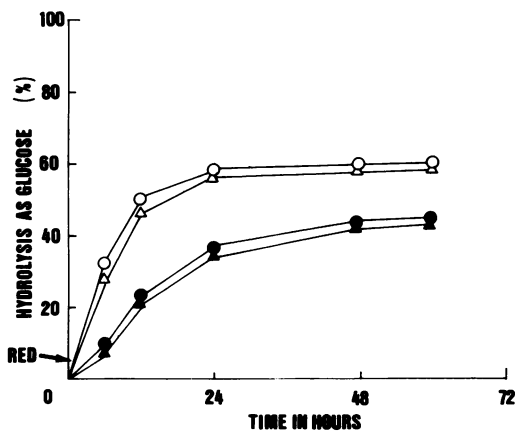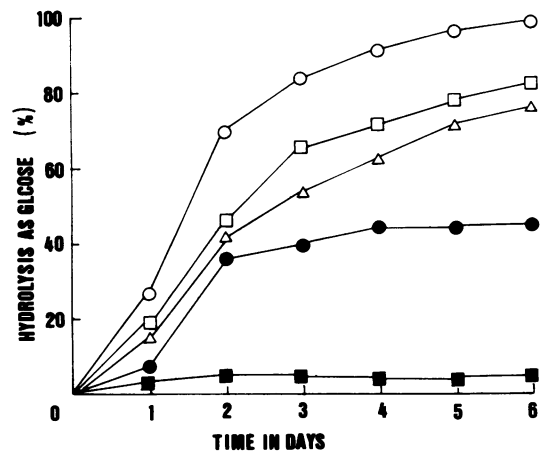

FIG. 8. Synergistic effects of raw-starch-adsorbable and raw-starch-unadsorbable α-amylases with *A. awamori* var. *kawachi* GA I. Symbols: ○, mixed preparation of raw-starch-adsorbable α-amylase and GA I; □, mixed preparation of raw-starch-unadsorbable α-amylase and GA I; △, GA I; ●, raw-starch-adsorbable α-amylase; ■, raw-starch-unadsorbable α-amylase. Reaction mixtures containing 300 mg of raw corn starch in 6 ml of 0.1 M acetate buffer (pH 5.2), 9 ml of mixed enzyme solution, and 33 ml of pure water were incubated at 30°C in a stationary state. A 6-ml portion of GA I (10 U/ml) plus 3 ml of raw-starch-adsorbable α-amylase (2.0 U/ml) or 3 ml of raw-starch-unadsorbable α-amylase (2.0 U/ml) was used as the mixed enzyme solution. The hydrolysis curves obtained were compared with that of the control (6 ml of GA I or 6 ml of raw-starch-adsorbable or raw-starch-unadsorbable α-amylase).

FIG. 7. Hydrolysis of various substrates. Symbols: ○, digestion curve of M-33 α-amylase with gelatinized potato starch; ●, glycogen; △, digestion curve of M-72 α-amylase with gelatinized potato starch; ▲, glycogen.
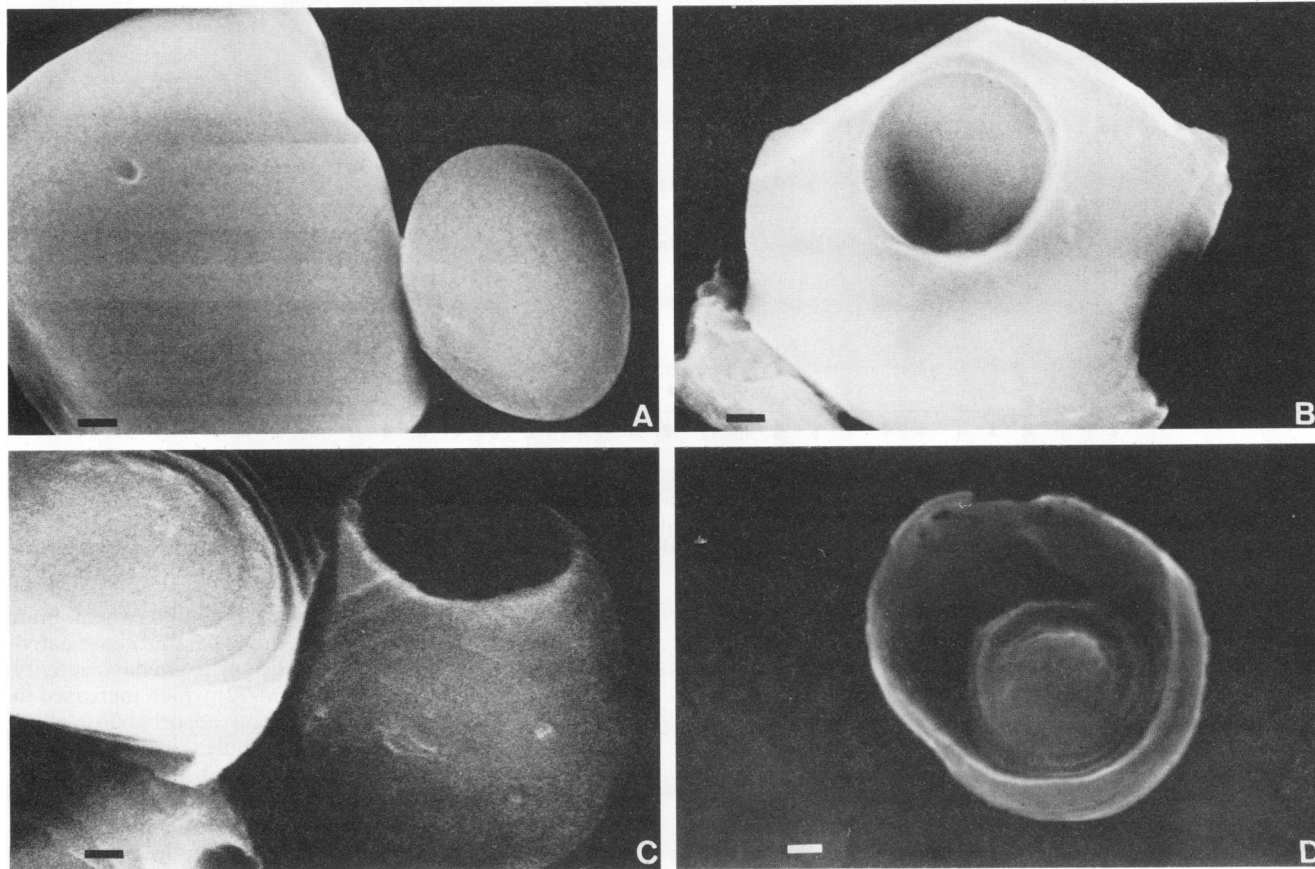
FIG. 9. Scanning electron micrograph of raw corn starch. Native corn starch granule: recovered from (A) 2 days or (B,C) 4 days of incubation. (D) A reaction mixture containing 300 mg of raw corn starch, 6 ml of 0.1 M acetate buffer (pH 5.2), 6 ml of raw-starch-adsorbable α-amylase, 36 ml of pure water, and 1 ml of toluene was incubated at 30°C in a stationary state and then centrifuged. The precipitates were collected and dried in a critical-point dryer (JEOL Ltd.) and used as the sample for scanning electron microscopy. Bars, 1 μm.

maximum amounts of M-33 α-amylase were obtained after 24 h. The culture filtrate of a 24-h culture of M-33 and that of a 38-h culture of M-72 were supplied for purification of enzymes.

Purification of mutant α-amylases. The lyophilized preparations of both culture filtrates were dissolved in 0.1 M acetate buffer (pH 6.0) and applied to a Sephacryl S-300 column. Fractions of α-amylase were combined and applied to a DEAE-Cellulofine AH column. The chromatographic pattern of M-33 α-amylase is shown in Fig. 2. The recovery and specific activity of M-33 α-amylase are summarized in Table 1.

Homogeneity of purified α-amylase. The purified M-33 and M-72 α-amylases were homogeneous on disc electrophoresis (Fig. 3).

Adsorbabilities of mutant α-amylases onto corn starch granules. Purified M-33 α-amylase was adsorbed more than 90% onto corn starch granules at pH 4.0. Purified M-72 α-amylase was not adsorbed onto corn starch granules at any pH values (Fig. 4).

Properties of mutant α-amylases. (i) MW. Logarithmic plots of reference proteins versus their relative mobilities are shown in Fig. 5. The MW of raw-starch-adsorbable α-amylase from mutant M-33 was estimated to be 88,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and that of raw-starch-unadsorbable α-amylase from mutant M-72 was estimated to be 54,000.

(ii) pH stability and optimal pH. M-33 α-amylase was stable at pH 4.0 to 9.0 and 90% activity remained at pH 2.0 (Fig. 6A). M-72 α-amylase was stable at pH 4.0 to 9.0 and 35% activity remained at pH 3.0; it was completely inactivated at pH 2.0. The optimal pH values of both enzymes were found to be pH 5.2 at 45°C (Fig. 6B).

(iii) Hydrolysis of various substrates. The hydrolysis curves exhibited by both enzymes for various amylaceous substrates are shown in Fig. 7. This shows that both enzymes hydrolyzed gelatinized potato starch and glycogen to limits of 60 and 40%, respectively. Sugar products in the reaction mixture were glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose. The hydrolysis degree at the erythro point in the iodine color reaction was 5.0%. Neither preparation showed maltase activity; glucose and maltose were produced from maltotriose.

(iv) Digestion of raw corn starch and electron microscopic observation of digested starch granules. Raw-starch-adsorbable α-amylase from mutant M-33 digested raw corn starch and completely solubilized the whole granules within 6 days (Fig. 8). The raw-starch-unadsorbable α-amylase from mutant M-72 did not show any digestibility on raw corn starch. The fine structure of these digested starch granule was observed with a scanning electron microscope. Figure 9 shows the corn starch granules of various extents of digestion by raw-starch-adsorbable α-amylase. The native starch granules are shown in Fig. 9A. The starting holes in the corn

starch digestion were observed at three to five points per granule (Fig. 9B). The holes and the digested surface of the starch granule revealed the characteristic layered structure (Fig. 9C). The holes gradually widened (Fig. 9D), and then they were completely solubilized within 6 days. No holes were observed on any corn starch granules with raw-starch-unadsorbable α-amylase from mutant M-72.

(v) **Synergistic effect with glucoamylase I in digestion of raw corn starch.** The raw-starch-digesting α-amylase from mutant M-33 exhibited a high synergistic effect with the glucoamylase I from *A. awamori* var. *kawachi* in the digestion of raw corn starch. The addition of raw-starch-digesting α-amylase led to twice the hydrolysis rate (Fig. 8). However, raw-starch-nondigesting α-amylase from mutant M-72 exhibited little synergistic effect.

DISCUSSION

To certify the presence of a novel type of fungal α-amylase, we tried to select mutants which could produce high amounts of the raw-starch-adsorbable, raw-starch-digesting α-amylase, according to the technique described in a previous paper (5) on the high productivity of raw-starch-adsorbable, raw-starch-digesting glucoamylase O in a protease-negative, glycosidase-negative mutant of *A. awamori* var. *kawachi*.

A purified preparation of α-amylase from *A. ficum* mutant M-33 was electrophoretically homogeneous, hydrolyzed gelatinized potato starch and glycogen up to 60 and 40%, respectively, and showed no maltase activity, and the hydrolysis degree at the erythro point in the iodine color reaction was 5.0%. These data supported the idea that this α-amylase preparation proved not to contain glucoamylase activity. We therefore concluded that the raw-starch-adsorbable α-amylase from mutant M-33 digested raw starch granules by itself. The purified preparation of α-amylase from mutant M-72 did not show adsorbability or digestibility on raw starch granules. However, this α-amylase showed digestibility on gelatinized potato starch and glycogen. Thus, the fungal α-amylases could be designated as raw-starch-digesting α-amylase I and raw-starch-nondigesting α-amylase II.

α-Amylase I from M-33 (MW 88,000) was higher in molecular weight than any other fungal α-amylase (MW 51,000 to 60,000) previously reported (2, 12, 16). α-Amylase II from mutant M-72 (MW 54,000) was almost identical to the above-mentioned typical fungal α-amylases. α-Amylase I was found to be more acid resistant and stable even at pH 2.0, similar to the acid-stable α-amylase of *A. niger* (11) and *A. aureus* (12), whereas α-amylase II, similar to the other ordinary fungal α-amylases, was completely inactivated at this pH. Both the α-amylase and glucoamylase from mutant M-33 showed the intensive raw starch digestibility at acidic pH and exhibited a high synergistic effect with each other in

the digestion of raw starches. We therefore expected that mutant M-33 would be useful as the saccharifying agent fortified with the novel type of α-amylase I for high-concentration ethanol fermentation of uncooked corn (8).

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