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Purification and Characterization of a Maltotetraose-Forming Alkaline α-Amylase from an Alkalophilic *Bacillus* Strain, GM8901

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An alkalophilic bacterium, *Bacillus* sp. strain GM8901, grown at pH 10.5 and 50°C, produced five alkaline amylases in culture broth. At an early stage of the bacterial growth, amylase I (Amyl I) was produced initially and then, as cultivation progressed, four alkaline amylases, Amyl II, Amyl III, Amyl IV, and Amyl V, were produced from proteolytic degradation of Amyl I. A serine protease present in the culture medium was believed to be involved in Amyl I degradation. We purified Amyl I from the culture supernatant by ammonium sulfate precipitation, heparin–Sepharose CL-6B column chromatography, phenyl-Toyopearl column chromatography, and Mono Q HR5/5 high-performance liquid chromatography. The molecular weight of Amyl I was estimated to be about 97,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amyl I had an extremely high optimal pH of 11.0 to 12.0 and was stable in a broad pH range of 6.0 to 13.0. Amyl I had an optimal temperature of 60°C and was stable up to 50°C. Thermostability was increased in the presence of Ca^{2+} and soluble starch. The enzyme required metal ions such as Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Ag^{+}, Zn^{2+}, and Fe^{2+} for its enzyme activity and was inhibited by 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. According to the mode of action of Amyl I on starch, Amyl I was classified as an α- and exo-amylase. Amyl I produced maltotetraose predominantly from starch via intermediates such as maltohexaose and maltopentaose.

Alkaline amylases that have optimum pH values higher than 8.0 have potential applications for hydrolyzing starch under high pH conditions in the starch and textile industries and as an ingredient in detergents for automatic dishwashers and laundries (6, 12, 13). They can also be used as model proteins for understanding the molecular basis of the alkalophilicity of the enzymes, which may be of value in protein engineering. Since Horikoshi (8) first reported an alkaline amylase of an alkalophilic *Bacillus* strain, A-40-2, alkaline amylases have been identified in *Bacillus* sp. strain NRRL B-3881 (2), *Bacillus* sp. strain H-167 (7), *Bacillus licheniformis* TCRDC-B13 (1), *Bacillus alcalothermophilus* A3-8 (13), and *Streptomyces* sp. strain KSM-9 (12). However, there is only a limited amount of information concerning the enzymatic and molecular biological properties of these alkaline amylases. Only one alkaline amylase gene has been cloned and sequenced, i.e., that from *Bacillus* sp. strain 707 (9, 25).

Previously, we isolated an alkalophilic *Bacillus* strain, GM8901, optimally grown at pH 10.5 and 50°C (20). Alkaline amylase produced from the bacterium has unique properties in that it produces predominantly maltotetraose from starch at an extremely high pH of 11.0 to 12.0. We thought that the alkaline amylase of *Bacillus* sp. strain GM8901 might be a good model enzyme for elucidating a molecular basis of alkalophilicity of the enzyme. In addition to this, the alkaline amylase can be utilized for the production of maltotetraose from starch, which has potential uses in the food, pharmaceutical, and fine-chemical industries because of its low sweetness, superior moisture retention, high viscosity and freezing point, and other special properties, compared with conventional sugar syrups (5). Although a maltotetraose-forming amylase has been discovered in *Pseudomonas stutzeri* (15, 16) and *Bacillus* strain MG-4 (21), our alkaline amylase is the first maltotetraose-forming enzyme with an optimal pH of 11.0 to 12.0 from alkalophilic bacteria. To better understand the alkaline amylase of *Bacillus* sp. strain GM8901, we describe here the purification and characterization of the enzyme. Recently, we cloned the gene encoding maltotetraose-forming alkaline amylase from *Bacillus* sp. strain GM8901 (19), and the DNA sequencing is in progress.

MATERIALS AND METHODS

Bacterial strain, medium, and culture conditions. Strain GM8901 was isolated as an alkaline amylase producer from soil on the shore of a river in Jangsung, Korea. The taxonomic study of the strain was done in our previous report (20). This strain belongs to the genus *Bacillus* and has a close resemblance to *B. licheniformis*. The medium used was composed of 10 g of soluble starch, 5 g of peptone (Difco), 5 g of yeast extract (Difco), 1 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 10.5 g of Na_2CO_3 (each per liter; pH 10.5). An inoculum was prepared by cultivating *Bacillus* sp. strain GM8901 for 1 day at 50°C in a rotary shaking incubator at 200 rpm. The main culture was done in a jar fermentor (MD300-5L; B. E. Marubishi Co., Tokyo, Japan) by inoculating 1% (vol/vol) of seed culture under the conditions of 50°C, 200 rpm, and 1 volume of air per working volume per min (vvm).

Purification of alkaline amylase. *Bacillus* sp. strain GM8901 was cultivated for 8 h in a jar fermentor containing 3 liters of medium at 50°C, 200 rpm, and 1 vvm. A total of 3 liters of bacterial culture was centrifuged at 6,000 × *g* for 20 min to remove the cells. The supernatant was brought to 80% ammonium sulfate saturation by adding solid ammonium sulfate to an ice bath. After the supernatant was left standing overnight at 4°C, the precipitate was collected by centrifuging at 15,000 × *g* for 30 min and dissolved in 10 mM sodium phosphate buffer (pH 8.0). The enzyme solution was dialyzed at 4°C against the same buffer for 1 day, with several changes of buffer. After removal of insoluble matter by centrifuging at 15,000 × *g* for 30 min, the enzyme solution was applied to a heparin–Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column (2.5 by 9 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0). The column was washed with 300 ml of equilibrating buffer. The enzyme elution was done at a flow rate of 0.6 ml · min^{-1} with a 200-ml linear KCl gradient (0 to 1.0 M). Fractions (2.0 ml per tube) were collected, and their alkaline amylase activity was determined as described below. The active fractions collected from the heparin–Sepharose CL-6B column were adjusted to 1.0 M ammonium sulfate by adding

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solid ammonium sulfate. Then, the enzyme solution was loaded on a phenyl-Toyopearl column (2.5 by 4 cm) equilibrated with 1.0 M ammonium sulfate–10 mM sodium phosphate buffer (pH 8.0). After washing with 100 ml of the same equilibration buffer, the enzyme was eluted by a descending gradient of ammonium sulfate (from 1.0 to 0.0 M) dissolved in 10 mM sodium phosphate buffer (pH 8.0). The active enzyme fractions collected from the phenyl-Toyopearl column were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) overnight with several changes of the buffer and concentrated with a Centricon 30 (Amicon Division, W. R. Grace & Co., Beverly, Mass.). The concentrated enzyme was applied to a Mono Q HR5/5 high-performance liquid chromatography (HPLC) column equilibrated with 10 mM Tris HCl buffer (pH 8.0). The enzyme was eluted serially with 5 ml of the buffer only, an NaCl gradient of 0.0 to 0.5 M (15 ml), and then an NaCl gradient of 0.5 to 1.0 M (50 ml). The active fractions obtained from the Mono Q HR5/5 HPLC column were dialyzed against distilled water and used for subsequent experiments.

Electrophoresis and molecular mass determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 15% polyacrylamide gel with the Mini Protean II electrophoresis system (Bio-Rad, Richmond, Calif.) as described by Laemmli (10). Proteins on the polyacrylamide gel were stained with 0.2% Coomassie brilliant blue R-250. Marker proteins with molecular weights ranging from 97,400 to 14,400 were used for estimation of the molecular weight of purified alkaline amylase. Native 12% PAGE was performed as described in the Bio-Rad instruction manual.

Activity staining of alkaline amylase in the native polyacrylamide gel. After electrophoresis, the native polyacrylamide gel was soaked for 30 min in 1% soluble starch in 50 mM glycine-NaOH buffer (pH 10.5) at 50°C with slow shaking. Amylase bands were visualized by staining the gel with a KI-I_2 solution (0.5% KI and 0.05% I_2).

Alkaline amylase assay. The routine enzyme assay used for alkaline amylase activity involved measuring the reducing sugars resulting from the hydrolysis of soluble starch. A 200-μl volume of appropriately diluted enzyme solution was mixed with 1 ml of 1% soluble starch in 50 mM glycine-NaOH buffer (pH 10.5), and the mixture was incubated for 30 min at 50°C. The amount of reducing sugars produced was determined by the dinitrosalicylic acid method (11), with glucose as the calibration standard. One unit of alkaline amylase activity is defined as the amount of enzyme which liberates 1 μmol of reducing sugars per min under the specified conditions.

Protein determination. Protein concentrations were determined by the Bio-Rad protein assay methods described by Bradford (3). Microassay procedures were performed, and bovine serum albumin was used as the standard protein.

Effects of pH and temperature. The relative alkaline amylase activity was determined at several pH values (4.0 to 13.0) with 50 mM citrate-sodium citrate buffer (pH 4.0 to 7.0), 50 mM Tris-HCl buffer (pH 7.0 to 9.0), 50 mM glycine-NaOH buffer (pH 9.0 to 11.0), 50 mM sodium phosphate-NaOH buffer (pH 11.0 to 12.0), and 50 mM hydroxide-chloride buffer (pH 12.0 to 13.0). The same buffers were used to determine the pH stability of the enzyme. Ten microliters of enzyme solution was mixed with 10 μl of 1 mM buffer, and the mixture was incubated for 1 h at 37°C. After cooling on ice, the samples were diluted 10-fold with 100 mM glycine-NaOH buffer (pH 10.5) and then used for determining the residual enzyme activities.

The optimal temperature was determined by assaying the enzyme activity at various reaction temperatures. To determine the thermostability, enzyme samples in 50 mM glycine-NaOH buffer (pH 10.5) were incubated at various temperatures, and then samples were withdrawn for enzyme assay at appropriate time intervals.

TLC. The reaction end products of alkaline amylase were analyzed by thin-layer chromatography (TLC) with a precoated silica gel plate (Kieselgel 60 F254; E. Merck AG, Darmstadt, Germany). After developing the products with a solvent system of butanol-acetic acid-water (3:1:1, vol/vol/vol), the spots were visualized by spraying with 20% sulfuric acid in ethanol and baking in a 120°C oven for 30 min.

HPLC. The purified enzyme was incubated at 50°C with 5% soluble starch in 50 mM glycine-NaOH buffer (pH 10.5). Samples were removed at different time intervals and heated in boiling water for 5 min to stop the reaction. The products were analyzed by HPLC (Gilson, Middleton, Wis.) on a μBondapak NH_2 column (3.9 by 300 mm; Millipore Corp., Bedford, Mass.) with a solvent of acetonitrile-water (70:30) as an eluant at a flow rate of 1 ml/min and quantified by a microprocessor equipped with data analysis software (Gilson 712 HPLC system controller software) in comparison with standard malto-oligosaccharides.

Identification of the anomeric form of the product. The mutarotation of the product from soluble starch with the purified enzyme was examined. The reaction was begun by mixing 0.5 ml of enzyme with 2.5 ml of 5% soluble starch in 50 mM glycine-NaOH buffer (pH 10.5) in a cuvette with an automatic digital polarimeter (Autopol III; Rudolph Research, Fairfield, N.J.). The change in optical rotation at 589 nm was recorded. After 30 min of reaction, 0.1 ml of 5% mercuric chloride was added to stop the reaction, and the mutarotation of the formed products was recorded.

Chemicals and reagents. Soluble starch was purchased from Shinyo Pure Chemicals Co. (Osaka, Japan); amylose, amylopectin, blue starch, pullulan, α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, maltose, malto-oligosaccharides, phenylmethylsulfonyl fluoride (PMSF), SDS, and EDTA were obtained from Sigma Chemical Co. (St. Louis, Mo.); size marker proteins of SDS-PAGE were obtained from Bio-Rad. The other chemicals used were of reagent grade.

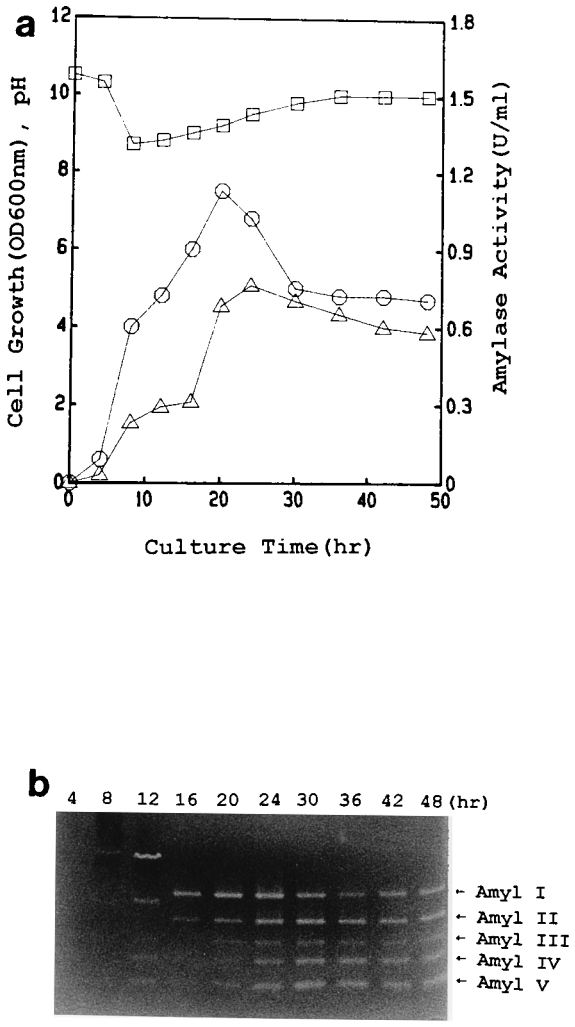


FIG. 1. Time courses of cell growth, amylase production, and pH change in culture medium. *Bacillus* sp. strain GM8901 was cultivated in a jar fermentor with a working volume of 3 liters under the initial conditions of pH 10.5, 50°C, 200 rpm, and 1 vvm. (a) Changes in cell growth (○), amylase production (△), and pH (□); (b) change of the isozyme pattern of alkaline amylase by culture time. Fifty milliliters of culture supernatant was concentrated fivefold by ammonium sulfate precipitation (80% saturation) and dialysis, and the concentrate was analyzed by native 12% PAGE and activity staining. OD, optical density.

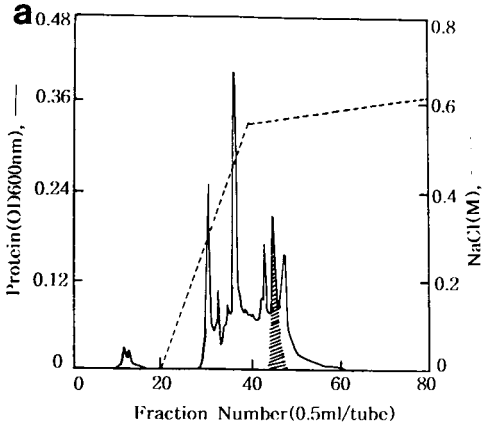
RESULTS

Amylase production and proteolytic degradation of the enzyme. As described in our previous paper (20), alkaline amylase from *Bacillus* sp. strain GM8901 is an extracellular enzyme induced by soluble starch and produced maximally at pH 10.5 and 50°C. During the cultivation of the bacterium in 3 liters of medium containing 1% soluble starch as a sole carbon source, the changes in cell growth, alkaline amylase activity, and pH were examined (Fig. 1a). The amylase activity increased with the increase of cell growth and reached a maximum level at 24 h (0.75 U ml^{-1}); thereafter, cell concentration and amylase activity decreased gradually. To gain information on the change of extracellular alkaline amylase pattern during cultivation, culture supernatants taken at various time intervals were concentrated fivefold with 80% saturation of ammonium

sulfate and separated on a native 12% polyacrylamide gel; alkaline amylases on the gel were then visualized by an activity staining method (Fig. 1b). From the sample taken at 8 h of cultivation, one major alkaline amylase was observed. However, thereafter, the number of alkaline amylases increased to five with the increase of culture time. We designated these alkaline amylases Amyl I, Amyl II, Amyl III, Amyl IV, and Amyl V. Amyl I and Amyl II of culture supernatant taken at 8 and 12 h appeared at much higher positions on the native gel than those of the other culture filtrates. The delayed migration of Amyl I and Amyl II was due to their high affinity to soluble starch present in the concentrates of culture supernatant (data not shown). The activity of Amyl I with the highest molecular weight showed a peak at 24 h of cultivation by native PAGE. However, after that time, the activity of Amyl I decreased gradually, and the activities of the other alkaline amylases, Amyl II, Amyl III, Amyl IV, and Amyl V, increased with time. When Amyl I, separated by electroelution from the native gel without the contamination of the other alkaline amylases, was incubated with a small amount of 24-h culture supernatant, it was converted into Amyl II, Amyl III, Amyl IV, and Amyl V (data not shown). This result suggested that Amyl II, Amyl III, Amyl IV, and Amyl V are produced from Amyl I, presumably by proteolytic degradation. We found an alkaline protease activity in the culture supernatant. The alkaline protease was partially purified from the culture supernatant by ammonium sulfate precipitation (80% saturation), phenyl-Toyopearl column chromatography, and Mono Q HR 5/5 HPLC (Fig. 2a). The partially purified protease was completely inhibited by PMSF, suggesting a subtilisin-like serine protease. As shown in Fig. 2b, this enzyme degraded Amyl I into Amyl II, Amyl III, Amyl IV, and Amyl V. This result clearly showed that Amyl II, Amyl III, Amyl IV, and Amyl V are produced from proteolytic degradation of Amyl I by an alkaline serine protease present in the culture supernatant.

Purification of alkaline amylase Amyl I. After 8 h of cultivation of *Bacillus* sp. strain GM8901, crude enzyme was obtained from the culture supernatant by ammonium sulfate precipitation (80% saturation) and dialysis. To minimize the proteolytic degradation of Amyl I by the serine protease, all of the purification steps were carried out at 4°C. PMSF, a serine protease inhibitor, could not be used for purification steps because it irreversibly inactivated Amyl I. Amyl I was purified serially by heparin–Sepharose CL-6B column chromatography, phenyl-Toyopearl column chromatography, and Mono Q HR 5/5 HPLC. The purification steps are summarized in Table 1. The final enzyme preparation had a specific activity of 157.5 U/mg of protein and gave a single protein band by SDS-PAGE with a molecular mass of about 97,000 (Fig. 3). The native polyacrylamide gel stained with the KI-I_2 solution showed a single alkaline amylase band which coincided with that of Amyl I. Through the purification steps described above, Amyl I was purified to homogeneity without detectable contamination of the other alkaline amylases produced from proteolytic degradation of Amyl I.

Effect of pH on the enzyme activity. The activity of Amyl I was assayed in buffers of various pH values, and the relative activities are shown in Fig. 4a. The optimum pH of Amyl I was 11.0 to 12.0. Amyl I shows high enzyme activity (above 90%) at an alkaline pH range of 10.5 to 12.0. However, at pH values below 9.0, the enzyme activity dropped sharply to below 50% of maximal activity. To examine the pH stability of Amyl I, the enzyme was incubated in buffers with various pH values for 1 h at 50°C, and the residual enzyme activity was assayed. As shown in Fig. 4b, Amyl I retained more than 85% of its initial enzyme activity at the pH range of 6.0 to 13.0. However, the



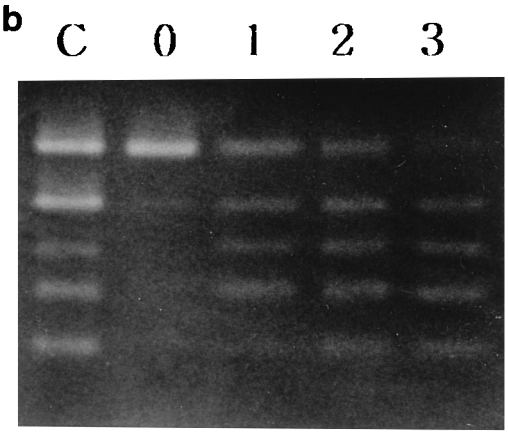


FIG. 2. Proteolytic degradation of an alkaline amylase (Amyl I) by an alkaline protease present in culture medium of *Bacillus* sp. strain GM8901. (a) Alkaline protease was partially purified from culture supernatant by ammonium sulfate precipitation (80% saturation), phenyl-Toyopearl column chromatography, and Mono Q HR5/5 HPLC. The Mono Q HR5/5 HPLC is shown. The alkaline protease peak is marked with a shadow. (b) Degradation of Amyl I into Amyl II, Amyl III, Amyl IV, and Amyl V by the partially purified alkaline protease. The purified Amyl I was incubated with the partially purified alkaline protease, and the reaction samples taken at 0 h (lane 0), 1 h (lane 1), 2 h (lane 2), and 3 h (lane 3) were analyzed by native 12% PAGE and activity staining. Lane C is the enzyme sample obtained from a 24-h culture supernatant described in the legend to Fig. 1b. OD, optical density.

pH stability of Amyl I was very low at pH values below 5.0 (80% and nearly 100% inactivations at pH 5.0 and 3.0, respectively). These results indicated that Amyl I is an extremely alkalophilic enzyme that has a high optimal pH of 12.0 and that shows high stability in the pH range of 6.0 to 13.0. To the best of our knowledge, such an alkaline amylase has not been reported to date. Amyl I can be used as a good model protein for investigating the molecular basis of alkalophilicity of moderately thermostable alkaline enzymes.

Effect of temperature on the enzyme activity. The enzyme activity of Amyl I, buffered at pH 10.5, was measured at various temperatures. As shown in Fig. 5a, Amyl I has an optimum temperature of 60°C. To examine the thermostability of Amyl I, the enzyme buffered at pH 10.5 was incubated at various temperatures and samples were taken at appropriate time in-

TABLE 1. Summary of the purification steps of alkaline Amyl I from the culture supernatant of *Bacillus* sp. strain GM8901[a]

Purification step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)
Culture supernatant	870	675	1.29	1.0
Ammonium sulfate precipitation (80% saturation) and dialysis	473	359	1.32	1.0
Heparin–Sepharose CL-6B chromatography	180	8.4	21.4	16.6
Phenyl-Toyopearl chromatography	139	1.9	74.5	57.8
Mono Q HR5/5 HPLC	57	0.06	157.5	122.0

[a] Purification was started with 3.0 liters of culture supernatant collected after cells had been cultivated for 8 h in a jar fermentor.

tervals to assay the residual activity (Fig. 5b). After 8 h of incubation at 40 and 50°C, Amyl I retained nearly 100 and 85% of its initial activity, respectively. However, only 37 and 12% remained after 2-h incubations at 60 and 70°C, respectively. By the addition of 1 mM $CaCl_2$, the thermostability of Amyl I at 60°C was distinctively enhanced: after 2-h incubations of the enzyme with and without 1 mM $CaCl_2$, 78 and 37% of the initial activity remained, respectively. However, the addition of 1 mM $CaCl_2$ could not stabilize the enzyme activity above 70°C. The thermostability of Amyl I also increased in the presence of 1% soluble starch: after 30-min incubations of the enzyme with and without 1% soluble starch at 60°C, 54 and 22% of the initial activity remained, respectively.

Effect of metal ions and chemical reagents. The activity of Amyl I buffered with 10 mM glycine-NaOH (pH 10.5) was assayed in the presence of various metal ions and chemical reagents. As shown in Table 2, Amyl I is activated in the presence of 1 mM Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, or Ag^{+} by 120 to 154%. The addition of 1 mM Zn^{2+} or Fe^{2+} does not significantly affect the enzyme activity. However, 1 mM Hg^{2+} completely inhibited the enzyme activity. When Amyl I was preincubated with 1 mM EDTA, the enzyme activity dropped to 18% of its initial activity (82% inhibition). The enzyme recovered its original activity by the addition of 2 mM Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Ag^{+}, or Fe^{2+} after the preincubation with 1 mM EDTA.

When the activity of Amyl I buffered at pH 10.5 was assayed in the presence of 1 mM PMSF, significant inhibition was not

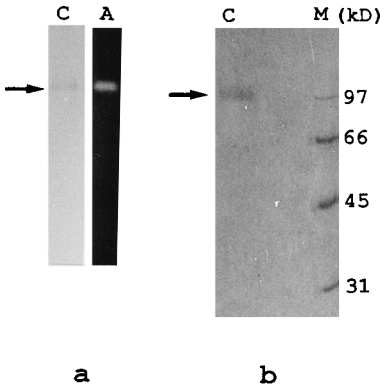


FIG. 3. Native PAGE (a) and SDS-PAGE (b) of the purified alkaline amylase (Amyl I). Amyl I was visualized by Coomassie brilliant blue staining (lanes C) and activity staining (lane A). Lane M, molecular mass markers (in kilodaltons). Arrows indicate the alkaline amylase. Protein concentrations were 2 μg (native PAGE) and 4 μg (SDS-PAGE).

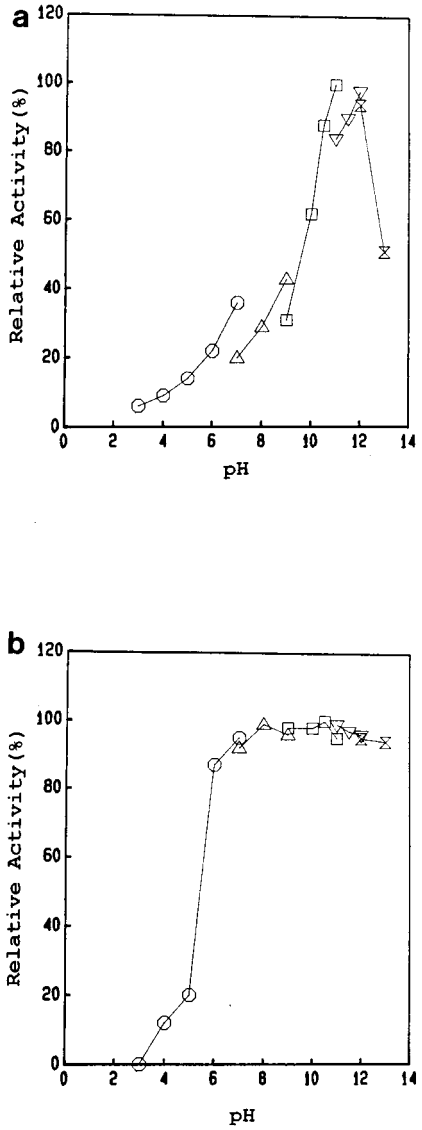


FIG. 4. Effect of pH on purified Amyl I activity (a) and stability (b). The buffers used were 50 mM citrate-sodium citrate buffer (pH 3.0 to 7.0) (○), 50 mM Tris-HCl buffer (pH 7.0 to 9.0) (△), 50 mM glycine-NaOH buffer (pH 9.0 to 11.0) (□), 50 mM sodium phosphate-NaOH buffer (pH 11.0 to 12.0) (▽), and 50 mM hydroxide-chloride buffer (pH 12.0 to 13.0) (⧖). For details, see Materials and Methods.

observed. However, with the increase of incubation time of Amyl I with 1 mM PMSF at 4°C, the enzyme inhibition was gradually increased: after 10 h of incubation, the residual enzyme activity of PMSF-treated sample was 24% of the nontreated enzyme activity. The addition of 0.1% detergent, including SDS, CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and Triton X-100, did not significantly affect the enzyme activity.

Mode of action of Amyl I on starch hydrolysis. Table 3 shows the relative activity of Amyl I on various substrates. Amyl I most easily hydrolyzed long-chain polysaccharides such as amylose and amylopectin and also easily hydrolyzed malto-oligosaccharides, including maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7). Short-chain malto-oligosaccharides such as maltose (G2), maltotriose (G3), and maltotetraose (G4) were resistant to Amyl I. Amyl I could not hydro-

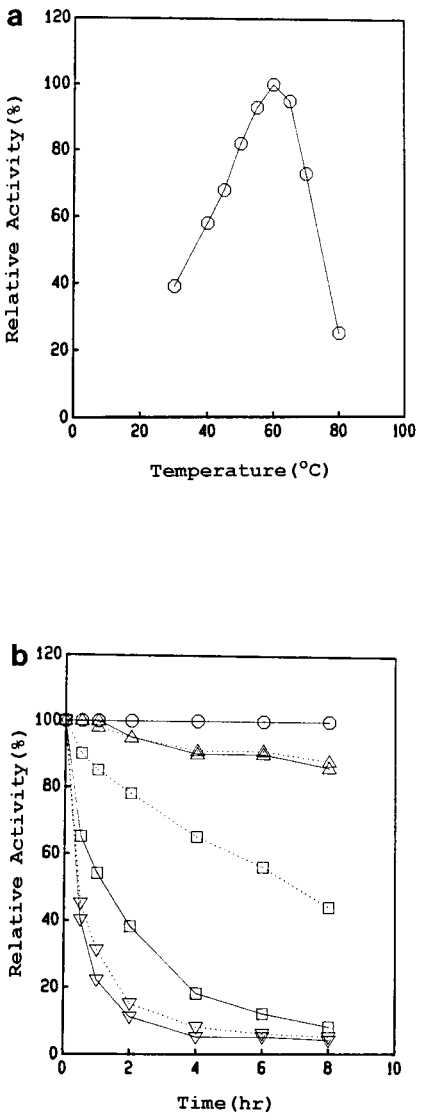


FIG. 5. Effect of temperature on purified Amyl I activity (a) and stability (b). For determining the thermostability, Amyl I was incubated at each temperature (i.e., 40°C [○], 50°C [△], 60°C [□], and 70°C [▽]) in the presence (dotted lines) or absence (solid lines) of 1 mM Ca^{2+}; after that, the remaining activity was measured. For details, see Materials and Methods.

lyze pullulan, an α-1,6-polysaccharide of G3, or α-, β-, and γ-cyclodextrins at all, even after overnight reaction. When Amyl I was incubated with 5% G5 or 5% G6 and the reaction products of samples taken at appropriate time intervals were analyzed by TLC, we could not detect any products formed via transglycosylation (data not shown). These results indicated that Amyl I hydrolyzes the α-1,4-glycosidic linkage of starch or preferably polysaccharides longer than G5, without glycosyl transferase activity or hydrolytic activity of the α-1,6-glycosidic linkage of starch.

To examine the mode of action of Amyl I on starch hydrolysis, amylose and malto-oligosaccharides, including G2 to G7, were treated with Amyl I, and the reaction products of samples taken at different time intervals were analyzed by TLC. As shown in Fig. 6, at an early stage of amylose hydrolysis, G6 was produced predominantly with the concomitant productions of a small amount of G4 and a trace amount of G5. After 2 h of

TABLE 2. Effect of metal ions on the activity of the purified Amyl I

Treatment	Relative activity (%)
None	100
Metal ion (1 mM)[a]	
$CaCl_2$	154
$AgNO_3$	138
$MgCl_2$	132
$CoCl_2$	129
$CuCl_2$	120
$ZnCl_2$	105
$FeCl_2$	95
$HgCl_2$	6
1 mM EDTA + metal ion (2 mM)[b]	
None	18
$CaCl_2$	145
$AgNO_3$	138
$MgCl_2$	135
$CoCl_2$	139
$CuCl_2$	140
$ZnCl_2$	105
$FeCl_2$	99

[a] The activity of Amyl I was assayed in the presence of a final concentration of metal ion of 1 mM.

[b] Amyl I was pretreated with 1 mM EDTA at 4°C for 2 h, and then enzyme activity was assayed in the presence of 2 mM metal ion or with no metal ion present.

reaction, a large amount of G4 and some G2 accumulated with the disappearance of G6 and G5. When 1% G2 or 1% G3 was treated with Amyl I at 50°C for 1 h, it did not produce any hydrolytic products on TLC (data not shown). G4 was also resistant to Amyl I but produced a trace amount of degradation products after 1 h of reaction. Malto-oligosaccharides such as G5, G6, and G7 were rapidly hydrolyzed into G4 by Amyl I via the intermediate of G5 (minor intermediate) or G6 (major intermediate) (Fig. 7). When we quantitatively analyzed reaction products of Amyl I from 5% soluble starch with HPLC, nearly the same results as those of TLC analysis were obtained. As shown in Fig. 8, at an early phase of hydrolysis (1 h of reaction), 1% (wt/wt) G1, 12% G2, 1% G3, 26% G4, 6% G5, and 54% G6 malto-oligosaccharides were produced. As starch hydrolysis progressed, G4 and G2 increased gradually and while G6 and G5, inversely, decreased. After 20 h of hydrolysis, malto-oligosaccharide compositions were 9% G1,

TABLE 3. Relative activities of purified Amyl I on various substrates[a]

Substrate	Relative activity (%)
Soluble starch	100
Amylose	125
Amylopectin	140
Pullulan	ND
Cyclodextrins (α, β, γ)	ND
Maltose	ND
Maltotriose	ND
Maltotetraose	ND
Maltopentaose	35
Maltohexaose	56
Maltoheptaose	75

[a] The activity of Amyl I was assayed on various substrates by the standard assay method described in Materials and Methods except that 0.1% malto-oligosaccharides was used. ND, not detectable.

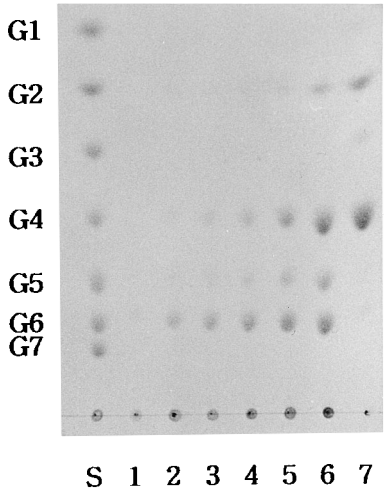


FIG. 6. TLC of the reaction products of purified Amyl I on amylose. Samples taken at 2 min (lane 1), 10 min (lane 2), 20 min (lane 3), 30 min (lane 4), 60 min (lane 5), 2 h (lane 6), and 15 h (lane 7) were analyzed by TLC. Lane S represents the standard sugars glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7).

27.6% G2, 8.3% G3, 53.2% G4, 1.3% G5, and 0.6% G6. These results indicated that Amyl I produced G4 as a major end product from starch through the intermediates of G6 (major intermediate) and G5 (minor intermediate).

TLC of reaction products of Amyl I on the various maltooligosaccharides described above (Fig. 6 and Fig. 7) suggested that Amyl I hydrolyzes starch by an exo-cleavage mode rather than an endo-cleavage mode. We examined various methods to determine whether Amyl I is an exo- or endo-type enzyme. When insoluble blue starch (starch azure; Sigma Chemical Co.), a substrate for colorimetric determination of endo-type α-amylases, was used as a substrate, Amyl I could not hydrolyze the blue starch at all. In addition to this, when Amyl I was reacted with 0.1% soluble starch and the blue values of the reaction mixture taken at appropriate time intervals were measured at 620 nm after blue color development with the final solution 0.5% KI–0.05% I_2–0.1 N HCl, the blue color of the reaction mixture diminished slowly as hydrolysis proceeded: only 8% of the blue value was reduced after 17% hydrolysis of the soluble starch. Percent hydrolysis was calculated by dividing the reducing sugar produced (calibrated with glucose as an internal standard) by the total reducing sugar obtained after a complete hydrolysis of 0.1% soluble starch. These results indicated that Amyl I follows an exo-cleavage mechanism. The exo-cleavage mechanism of Amyl I is also supported by the fact that Amyl I could not hydrolyze α-, β-, and γ-cyclodextrins. It has been reported that exo-amylases such as glucoamylase, β-amylase, and maltohexaose-forming amylase from *Klebsiella pneumoniae* could not hydrolyze the cyclodextrins but endo-amylases such as Taka-amylase and bacterial saccharifying amylase could (16).

The anomeric form of the products of Amyl I was examined with a polarimeter. Optical rotation decreased sharply after the reaction stopped (data not shown). This showed that the product has an α-configuration. Hence, our Amyl I was classified as an exo-α-amylase.

DISCUSSION

We purified Amyl I from an 8-h culture supernatant by ammonium sulfate precipitation, heparin–Sepharose CL-6B column chromatography, phenyl-Toyopearl column chromatography and Mono Q HR5/5 HPLC. Amyl I has a molecular mass of about 97,000. Amyl I has unique properties in that its optimal pH is extremely high (pH 11.0 to 12.0) and this enzyme is stable in a broad pH range (pH 7.0 to 13.0), requires metal ions for enzyme activity, is inhibited by 1 mM EDTA and 1 mM PMSF, and produces predominantly G4 from starch through intermediates such as G6 and G5. On the basis of its mode of action, Amyl I was classified as an exo-α-amylase.

Most of the *Bacillus* amylases have optimum pH values of from 5.0 to 8.0 (14, 17, 18, 22, 24), and an acidic amylase from *Bacillus acidocaldarius* has an optimum pH of 3.5 (4). Alkaline amylases reported previously had optimum pH values of 9.0 to 10.5 (1, 2, 8, 12, 13). However, our alkaline amylase Amyl I has an extremely high optimal pH value of 11.0 to 12.0, which to our knowledge is higher than that of any other amylases reported to date. Amyl I is stable in an alkaline pH range of 7.0 to 13.0: more than 90% enzyme activity remained after 1 h of incubation in this pH range. These results indicated that Amyl I is well adjusted to catalyze starch hydrolysis under extremely

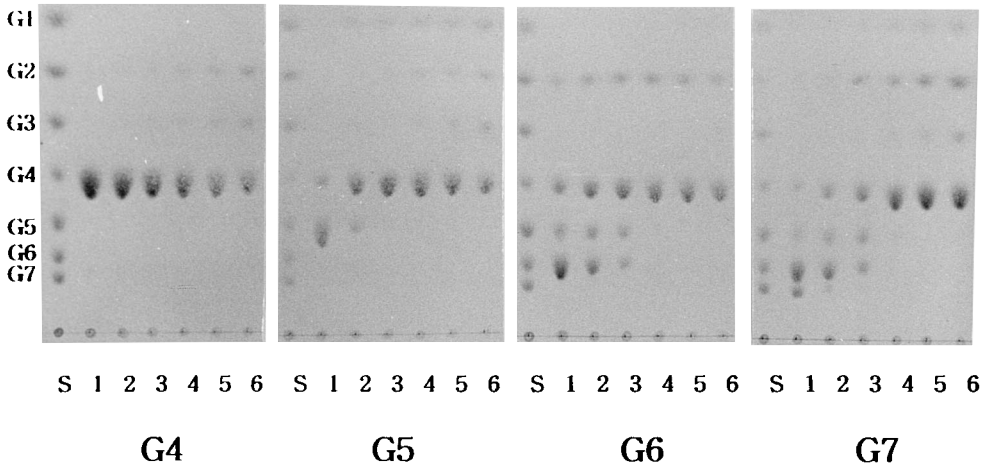


FIG. 7. TLC of the reaction products of purified Amyl I on maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7). Samples taken at 5 min (lanes 1), 10 min (lanes 2), 20 min (lanes 3), 30 min (lanes 4), 40 min (lanes 5), and 60 min (lanes 6) were analyzed by TLC. Standard sugars (lanes S) are as described in the legend to Fig. 6.

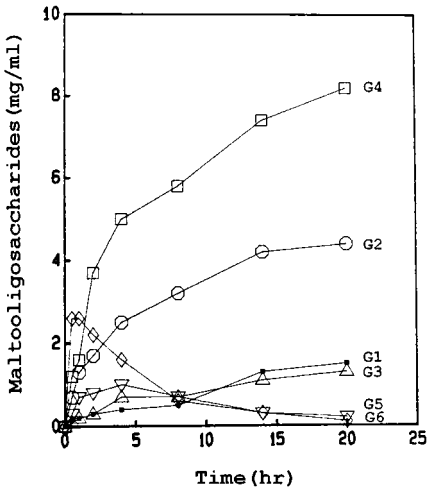


FIG. 8. Time course of malto-oligosaccharide production from 5% soluble starch by purified Amyl I. Samples taken at appropriate time intervals were analyzed by HPLC as described in Materials and Methods. Symbols (G1 to G6) are as described in the legend to Fig. 6.

alkaline conditions. It has been known that all enzymes of the α-amylase family are considered to catalyze the same basic reaction, a nucleophilic double-displacement mechanism with a transient covalent intermediate: two acidic amino acid residues (Asp or Glu) of protein are essentially involved in catalysis, i.e., one has an ionized carboxylic acid group and the other has an un-ionized carboxylic acid group (23). However, it is doubtful whether Amyl I can have an un-ionized carboxylic acid group in an active center even at pHs above 11.0 because the deionization of the carboxylic acid group of Asp and Glu residues of proteins is expected under this pH condition. With respect to this view, compared with other neutral and acidic amylases, Amyl I is believed to have a unique structure and reaction mechanism to catalyze starch hydrolysis under extremely high pH conditions. However, there are no reports describing the catalytic mechanism of alkaline amylases and the structure-function relationship of the enzymes. Further study of Amyl I will reveal the molecular basis of the alkalophilicity of the enzyme.

Amyl I has an optimal temperature of 60°C and is stable up to 50°C. Its thermostability is enhanced in the presence of Ca^{2+} and soluble starch. The enzyme is moderately thermostable when compared with other thermostable amylases of *Bacillus* sp. (3, 17, 19, 20). In our previous taxonomic study of *Bacillus* sp. strain GM8901, this strain has a close resemblance to *B. licheniformis*. Two types of amylase have been reported from *B. licheniformis*: one is thermostable alkaline amylase (1), and the other is thermostable alkali-tolerant amylase (17). Both enzymes have similar properties in pH and temperature effect: their optimal pH values are about 5.5 to 10.0 for the former and 5.0 to 8.0 for the latter, they are stable in the pH range of 6.0 to 11.0, and they have high optimal temperatures, 90°C for the former and 75°C for the latter. Our Amyl I showed a broad pH stability, similar to that of the two amylases of *B. licheniformis* described above. However, the optimal pH, optimal temperature, thermostability, and reaction end products of Amyl I are quite different from those of *B. licheniformis.*

The activity of Amyl I is greatly influenced by metal ions. Amyl I is activated by 1 mM Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, or Ag^{+} by 120 to 154%. The addition of 1 mM Zn^{2+} or Fe^{2+} does not significantly affect the enzyme activity. Amyl I is inhibited by a chelating agent, EDTA, and its full activity is recovered by adding Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Ag^{+}, and Fe^{2+}. This result suggested that metal ions are essential for the enzyme activity of Amyl I and that Amyl I does not show a strict specificity for metal ions. Ca^{2+} not only activates the activity of Amyl I but also enhances its thermostability. It has been reported that alkaline amylases of *Bacillus* sp. strain A-40-2 (8), *Bacillus* sp. strain NRRL B-3881 (2), and *B. alcalothermophilus* A3-8 (13) are stable in response to EDTA treatment, while the neutral amylases of *Bacillus amyloliquefaciens*, Taka-amylase A, and the liquefying amylase of *Bacillus subtilis* are sensitive to EDTA treatment (24).

Amyl I shows unique properties in the catalytic mode of action. Amyl I produces predominantly maltotetraose from soluble starch via the intermediates of maltohexaose (major intermediate) and maltopentaose (minor intermediate). Previously, maltotetraose-forming amylases were discovered in *P. stutzeri* (15, 17) and *Bacillus circulans* MG-4 (21). However, these enzymes showed their optimal pH to be in neutral range. Maltotetraose-forming alkaline amylase from alkalophilic bacteria has not been reported to date. Amyl I is the first alkaline amylase producing maltotetraose from starch as a major end product. On the basis of its mode of action, Amyl I can be classified as an exo- and α-amylase. It has been known that most α-amylases are endo-type enzymes that randomly hydrolyze starch. However, Amyl I is a rare α-amylase that hydrolyzes starch by an exo-cleavage mechanism.

Further molecular biological study of Amyl I will contribute to an understanding of the molecular basis of alkalinity of the enzyme, which may be of value in carbohydrate enzyme engineering. Besides this, Amyl I can also be applied to the production of maltotetraose from starch for use in nutrient foods for infants and aged persons (5).

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