Novel α-Amylase That Is Highly Resistant to Chelating Reagents and Chemical Oxidants from the Alkaliphilic Bacillus Isolate KSM-K38

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A novel α-amylase (AmyK38) was found in cultures of an alkaliphilic Bacillus isolate designated KSM-K38. Based on the morphological and physiological characteristics and phylogenetic position as determined by 16S ribosomal DNA sequence and DNA-DNA reassociation analysis, it was suggested that the isolate was a new species of the genus Bacillus. The enzyme had an optimal pH of 8.0 to 9.5 and displayed maximum catalytic activity at 55 to 60°C. The apparent molecular mass was approximately 55 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the isoelectric point was around pH 4.2. This enzyme efficiently hydrolyzed various carbohydrates to yield maltotriose, maltohexaose, and maltotetraose, and, in addition, maltose as major end products after completion of the reaction. The activity was not prevented at all by EDTA and EGTA at concentrations as high as 100 mM. Moreover, AmyK38 was highly resistant to chemical oxidation and maintained more than 80% of its original activity even after incubation for 1 h in the presence of excess H$_2$O$_2$ (1.8 M).

Starch, a main component of our daily diet, is frequently found not only in food residues on dishes but also in food stains on clothes (36). Enzymatic hydrolysis of starch is catalyzed by α-amylase (1,4-α-D-glucan glucoamylase; EC 3.2.1.1), β-amylase (1,4-α-D-glucan glucoamylase; EC 3.2.1.2), glucoamylase (1,4-α-D-glucan glucoamylase; EC 3.2.1.3), α-glucosidase (1,4-α-D-glucan glucoamylase; EC 3.2.1.20), and debranching enzymes such as pullulanase (pullulan 6-glucanohydrolase; EC 3.2.1.41) and isoamylase (glycogen 6-glucanohydrolase; EC 3.2.1.68). These amylolytic enzymes, especially α-amylase and pullulanase, are very important, particularly in the food and detergent industries (1, 28). We have found and characterized some unique debranching enzymes, such as a high-alkaline pullulanase (4), an alkali-resistant neopullulanase (16), an alkaline isoamylase (6), and an alkaline amyloliquifaciens (BAA) (23), and Bacillus stea thermophilus (BSA) (23), are acid or neutral enzymes having pH optima at around 6.5. These neutral enzymes are essentially useless in detergents because the working pH range of detergents is between 8 and 11 (21). Since Horikoshi (13) reported the first alkaline amylase from alkaliphilic Bacillus sp. strain A-40-2 in 1971, many alkaline amylases have been found in cultures of alkaliphilic Bacillus strains (14). Most of the alkaline amylases from these alkaliphilic bacilli reported to date are exo-type amylases, which are not suitable for use in detergents. Generally, α-amylases are metalloenzymes that contain at least one activating and stabilizing Ca$^{2+}$ ion (37, 39). It is well known that amylases are often inhibited by chelating reagents, such as zeolites, EDTA, and EGTA.

Recently, we found a novel α-amylase (AmyK) from cultures of the alkaline amyloliquifaciens producer alkaliphilic Bacillus sp. strain KSM-1378 (17) and succeeded in hyp.expressing the amyK gene in Bacillus subtilis cells (20). This enzyme is highly active at alkaline pH compared with BAA, BSA, and BSA. Furthermore, we improved the thermostability of AmyK by deletion of the Arg181-Gly182 residues (18) and substitution of a proline in the enzyme molecule (19). The deletion mutant enzyme also acquired resistance to chelating reagents such as EDTA and EGTA, but the resistance was still lower than that of BLA (unpublished data). In this paper, we report the isolation of a novel α-amylase (AmyK38) from cultures of alkaliphilic Bacillus sp. strain KSM-K38. AmyK38 is an alkaline α-amylase having high resistance to chelating reagents and chemical oxidants. Furthermore, this strain was suggested to be a novel species of Bacillus, as judged by 16S ribosomal DNA (rDNA) gene sequence and DNA-DNA hybridization analysis.

MATERIALS AND METHODS
Organism and culture conditions. The organism used was Bacillus sp. strain KSM-K38, which was originally isolated from a soil sample collected in Tochigi,
Enzyme assay.

Enzyme purification was done below 5°C. The centrifugal supernatant of the culture broth was treated with ammonium sulfate, and the fraction that precipitated at 80% saturation was collected. Precipitated forms were dissolved in a small volume of 10 mM Tris- HCl buffer (pH 7.0), and the solution was dialyzed overnight against 250 volumes of the same buffer. The retentate was then applied to a column of DEAE-Toyopearl 650 M (5 by 18 cm; Tosoh, Tokyo, Japan) that had been equilibrated with 10 mM Tris- HCl buffer (pH 7.0) and was then washed with 100 volumes of the same buffer, and proteins were eluted with a 3.0-liter linear gradient of 0.3 to 1.0 M NaCl in the same buffer, and proteins were eluted with a 3.0-liter linear gradient of 0.3 to 1.0 M NaCl in the same buffer, at a flow rate of 8.6 ml min\(^{-1}\). The active fractions were combined and concentrated by ultrafiltration (PM-10, 10,000 M cutoff; Millipore, Bedford, Mass.). The concentrated absorbance at 280 nm. The active fractions were combined and concentrated by ultrafiltration (PM-10, 10,000 M cutoff; Millipore, Bedford, Mass.). The concentrated absorbance at 280 nm.

Enzyme activity was determined usingEmail: a-amylase activity was measured using a mglucose per min. The reaction mixtures contained 1% (wt/vol) soluble starch (from potato; Sigma) in 10 mM potassium phosphate buffer (pH 8.0). The samples were removed at intervals and heated immediately in boiling water for 5 min to terminate the reaction. The products were analyzed by HPLC with a carbohydrate column (4.6 by 250 mm; Waters, Milford, Mass.) with acetonitrile-water (70:30, vol/vol) as eluent at a flow rate of 1.4 ml min\(^{-1}\), and they were measured with data analysis software, 805 Data Station (Waters), using authentic maltodextrins.

Analysis of anomic configuration. The anomic configuration of products of soluble starch hydrolyzed by the purified AmyK38 was determined by measuring the optical rotation of the hydrolysate (15). A reaction mixture (1 ml), consisting of 1% (wt/vol) soluble starch (from potato; Sigma) in 10 mM potassium phosphate buffer (pH 8.0), and enzyme (4.7 U ml\(^{-1}\)) was placed in a cuvette with a 5.0-cm light path. The change in optical rotation of the mixture was observed at room temperature for a high sensitivity SEPA-200 polarimeter (Horiba, Tokyo, Japan) by using the sodium line (589 nm). The rotatory power of the hydrolysate was observed by adding 2 drops of 28% ammonium solution after the optical rotation had become approximately constant.

Analysis of calcium in the AmyK38 molecule. The enzyme samples were dissolved overnight in 10 mM glucose- NaOH buffer (pH 10) at 5°C. The resultant reagent and dialysate portions were hydrolyzed with nitric acid and heated for 30 min. The active fractions were combined and concentrated by ultrafiltration (PM-10, 10,000 M cutoff; Millipore, Bedford, Mass.). The concentrated absorbance at 280 nm. The active fractions were combined and concentrated by ultrafiltration (PM-10, 10,000 M cutoff; Millipore, Bedford, Mass.). The concentrated absorbance at 280 nm.

Nucleotide sequence accession number. The 16S rDNA sequence data of AmyK38 have been submitted to the DDBJ, GenBank, and EMBL data banks with the accession no., AB044748. An extensive search of the scientific literature (PubMed: http://www.ncbi.nlm.nih.gov/PubMed) and databases (nr-aa, PIR, and Swiss-Prot) was performed to collect the 16S rDNA sequences of Bacillus strains using the BLAST2 program (2). Sequences incorporated in the present study are under the following accession numbers: B. agaradhaerens DSM 87217, X76445; B. clarkii DSM 87207, X76444; Bacillus halodeni DM 87207, X76449; Bacillus halodeni ATCC 27557, AB201187; Bacillus halodenitricans ATCC 49067, AB201186; Bacillus homohiosus DSM 87157, X76439; Bacillus pseudocalphatus DSM 87257, X76449; Bacillus halodenu triticans ATCC 49067, AB201187; Bacillus homohiosus DSM 87157, X76439; Bacillus pseudocalphatus DSM 87257, X76449; and Bacillus niacini IFO 15567, AB201194.

RESULTS AND DISCUSSION

Taxonomic characteristics of strain KSM-K38. The physiological and biochemical characteristics of strain KSM-K38 were identified. The organism was capable of growing over a
Bacillus pseudofirmus DSM 8715 T
Bacillus pseudocaldophilus DSM 8725 T
Bacillus alcalophilus DSM 485 T
Bacillus horikoshi DSM 8719 T
Bacillus halodurans ATCC 27597 T
Bacillus agaradhaerens DSM 8721 T
Bacillus clarkii DSM 8720 T
Bacillus sp. strain KSM-K38
Bacillus niacinicus IFO 13566 T
Bacillus halotolerans DSM 8723 T
Bacillus halodermophilus ATCC 49067 T

FIG. 1. Unrooted phylogenetic tree based on the 16S rDNA sequences of strain KSM-K38 and representative Bacillus strains. The searched sequences having similarity with less than 90.5% identity were omitted from the figure. The numbers at internal nodes are the percentages of bootstrap values derived from 1,000 samples in which the group to the right of the node was monophyletic. Bootstrap probability values less than 50% were omitted from the figure. Bar = 0.01 K_{nucl} unit, representing 0.01 inferred substitutions per nucleotide position. T, type strain (see Materials and Methods).

pH range from 9 to 11, but no growth was observed at pH 7. The range of temperature for growth was between 15 and 40°C with optimal growth around 30°C. It was a strict aerobe that was spore forming (cylindrical, central, or subterminal endospores), gram positive, motile, rod shaped (1.0 to 1.2 by 1.8 to 3.8 μm), and peritrichous. It was positive for production of catalase and oxidase and hydrolysis of starch, casein, gelatin, Tween 40, and Tween 60 and was negative for formation of catalase and oxidase and hydrolysis of starch, casein, gelatin, D-xylose, D-ribose, L-arabinose, D-mannitol, glycerol, sucrose, lactose, maltose, melibiose, trehalose, and D-raffinose but not on inositol, D-sorbitol, esculin, and rhamnose. Thus, this isolate is an obligately alkaliphilic Bacillus strain. The doubling time was 40 min when the organism was grown at 30°C in the soluble starch medium described above.

For further characterization of strain KSM-K38, we constructed a phylogenetic tree based on comparison of the 16S rDNA gene sequence of this strain and those of 10 type strains of Bacillus spp., as shown in Fig. 1. Similarity values were as low as 90.5 to 95.5% compared with these Bacillus strains. The sequence of strain KSM-K38 had the closest match (95.5% homology) with that from B. agaradhaerens. The next highest similarity was with B. clarkii (94.8% homology). The DNA-DNA hybridization of strain KSM-K38 with B. clarkii and B. agaradhaerens revealed a low association (less than 23%), as shown in Table 1. Moreover, the G+C contents of the DNA of strain KSM-K38, B. clarkii, and B. agaradhaerens were 46.2, 42.0, and 38.2 mol%, respectively. On the basis of the results of the phenotypic characteristics, the G+C content of genomic DNA, the 16S rDNA similarity, and the level of DNA-DNA hybridization, Bacillus sp. strain KSM-K38 is not closely related to any of the strains of Bacillus compared. Thus, we suggest that the isolate is a new species of the genus Bacillus.

### TABLE 1. Levels of relatedness between Bacillus sp. strain KSM-K38 and related Bacillus strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>% DNA-DNA hybridization with strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacillus sp. strain KSM-K38</td>
</tr>
<tr>
<td></td>
<td>Bacillus agaradhaerens DSM 8721 T</td>
</tr>
<tr>
<td></td>
<td>Bacillus clarkii DSM 8720 T</td>
</tr>
<tr>
<td>Bacillus sp. strain KSM-K38</td>
<td>96.2</td>
</tr>
<tr>
<td>Bacillus agaradhaerens</td>
<td>38.2</td>
</tr>
<tr>
<td>Bacillus clarkii</td>
<td>42.0</td>
</tr>
</tbody>
</table>

**NT**, not tested.

### TABLE 2. Purification of amylase produced by Bacillus sp. strain KSM-K38

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total act (U)</th>
<th>Sp act (U mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>243</td>
<td>1,254</td>
<td>5</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>80% ammonium sulfate precipitation</td>
<td>120</td>
<td>789</td>
<td>7</td>
<td>63</td>
<td>1.3</td>
</tr>
<tr>
<td>DEAE-Toyopearl chromatography</td>
<td>0.20</td>
<td>569</td>
<td>2,917</td>
<td>45</td>
<td>565.0</td>
</tr>
<tr>
<td>Toyopearl HW-55F chromatography</td>
<td>0.04</td>
<td>168</td>
<td>4,221</td>
<td>13</td>
<td>817.6</td>
</tr>
</tbody>
</table>

Substrate specificity. The purified enzyme was examined for its ability to hydrolyze various carbohydrates under the standard conditions of the assay, as shown in Table 3. Of the...
substrates tested, soluble starch was hydrolyzed by the enzyme, and amylopectin, glycogen, amylose, and dextrin were also hydrolyzed to a lesser extent. No reaction was observed with dextran; pullulan; or α-, β-, and γ-cyclodextrins. The product pattern of the purified enzyme with soluble starch (0.5% [wt/vol]) as substrate was examined by HPLC. The major products were maltotriose (G3) and maltohexaose (G6), with intermediate products being maltose (G2) and maltoheptaose (G7), as shown in Fig. 3. The typical molar ratios of products at equilibrium reached after 20 h were as follows: G7, 0.19 mM; G6, 0.49 mM; maltopentaose (G5), 0.10 mM; maltotetraose (G4), 0.09 mM; G3, 0.49 mM; G2, 0.31 mM; glucose (G1), 0.06 mM. This hydrolysis pattern was consistent with those of endo-type amylases. The anomeric configuration of the products was determined by measurement of optical rotation of the hydrolysate, as shown in Fig. 4. An abrupt downward shift of optical rotation occurred upon addition of ammonia solution after a 12-min incubation. This indicates that the hydrolysis products have an α-anomeric configuration, and hence, the enzyme is classified as an α-amylase.

**Effect of pH on activity and stability.** The effect of pH on the activity of AmyK38 was examined with soluble starch as the substrate in 50 mM buffers (acetate, pH 3.5 to 6.0; potassium phosphate, pH 6.0 to 8.0; glycine-NaOH, pH 9.0 to 10.5; carbonate, pH 10.0 to 12.0). The purified enzyme showed catalytic activity from pH 5.5 to 10.5 and was an alkaline enzyme, having a pH optimum of 8.0 to 9.5 in the buffers. More than 50% of the maximum activity was detectable between pH 6.5 and 10.

**TABLE 3. Substrate specificity of the purified enzyme**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch (potato)</td>
<td>100</td>
</tr>
<tr>
<td>Starch (sweet potato)</td>
<td>100</td>
</tr>
<tr>
<td>Starch (corn)</td>
<td>105</td>
</tr>
<tr>
<td>Amylose (corn)</td>
<td>65</td>
</tr>
<tr>
<td>Amylopectin (potato)</td>
<td>74</td>
</tr>
<tr>
<td>Glycogen (bovine muscle)</td>
<td>58</td>
</tr>
<tr>
<td>Pullulan</td>
<td>1</td>
</tr>
<tr>
<td>Dextran</td>
<td>0</td>
</tr>
<tr>
<td>Dextrin (corn)</td>
<td>27</td>
</tr>
<tr>
<td>α-Cyclodextrin</td>
<td>0</td>
</tr>
<tr>
<td>β-Cyclodextrin</td>
<td>0</td>
</tr>
<tr>
<td>γ-Cyclodextrin</td>
<td>0</td>
</tr>
</tbody>
</table>

*Added at 0.5% (wt/vol).

b The activity was measured at 50°C and at pH 10.0 in 50 mM glycine-NaOH buffer. The values shown are the percentages of the activity obtained using soluble starch as substrate, which is taken as 100%.

**FIG. 3. Analysis of products of hydrolysis of soluble starch by HPLC.** The reaction (0.3 U ml⁻¹) was done at 30°C and at pH 8.0 in 10 mM potassium phosphate buffer. Samples were taken at the indicated intervals and boiled for 5 min to terminate the reaction. The products were analyzed by HPLC, as described in Materials and Methods. Symbols used: ○, G1; ■, G2; △, G3; □, G4; ▼, G5; ●, G6; ◇, G7.

**FIG. 4. Optical rotation of the action of the purified enzyme with soluble starch.** The symbols indicate the optical rotations before (●) and after (○) addition of alkali to the digests, as described in Materials and Methods.
At pH 9, the specific activity of AmyK38 is approximately fivefold greater than that of BLA (17). Ca\(^{2+}\) ion (1 mM) inhibited the AmyK38 activity by 25 to 30% over a range from pH 8.0 to 10 (data not shown). To examine the pH stability of the purified enzyme, the enzyme (2.0 U ml\(^{-1}\)) was preincubated at the indicated pH in 10 mM Britton-Robinson buffer and at 40°C for 30 min, and then samples (0.1 ml) were used to measure the residual activity under the standard conditions of the assay. The enzyme was very stable, with more than 80% of the original activity detected over the wide range of pHs from 6 to 11.

**Effect of temperature on activity and stability.** The activity of AmyK38 was measured at various temperatures at pH 10 in 50 mM glycine-NaOH buffer. The alkaline enzyme showed catalytic activity from 20 to 80°C, and the optimal temperature was around 55 to 60°C. To examine the temperature stability of the enzyme, the time course of the thermal inactivation of the enzyme was monitored at pH 10 in 50 mM glycine-NaOH buffer. The enzyme retained full activity after 60 min of incubation at 30°C but lost 80% of the original activity after 30 min of incubation at 50°C in the absence of Cu\(^{2+}\) ions. This divalent cation (1 mM) did not protect it from the thermal inactivation of the enzyme at all. BLA was very stable under the same conditions (data not shown).

**Effects of metal ions and laundry surfactants.** AmyK38 was incubated with various metal ions (1 mM each) for 30 min at 30°C and at pH 10 in 50 mM glycine-NaOH buffer, and the residual activity was measured. Mn\(^{2+}\) ions inhibited the activity by 20%. Other metal ions, including Al\(^{3+}\), Fe\(^{3+}\), Ca\(^{2+}\), Co\(^{2+}\), Hg\(^{2+}\), Ag\(^{+}\), Cu\(^{2+}\), Ni\(^{2+}\), Fe\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), Ba\(^{2+}\), Be\(^{2+}\), Pb\(^{2+}\), Sr\(^{2+}\), Na\(^{+}\) (50 mM), and K\(^{+}\) (50 mM), were without effects on the activity. The enzyme was quite stable to incubation at 40°C for 1 h with various surfactants (0.1% [wt/vol] each), such as SDS, polyoxyethylene alkyl sulfate, polyoxyethylene alkyl ether, sodium α-sulfonated fatty acid ester, and alkyl glucoside. Linear alkylbenzene sulfonate and alkyl sulfate slightly inhibited the activity. These properties, together with the above results, of AmyK38 fulfill the essential requirements for enzymes that can be used as effective additives in laundry and automatic dishwashing detergents.

**Effects of chemical oxidants and chelating reagents.** Inactivation by chemical oxidation has been reported previously for an α-amylase from *B. subtilis* (26), as in the cases of an alkaline protease (subtilisin) (32) and various proteins and peptides (8). The oxidative stability of AmyK38 was then examined by measuring the residual activities after incubation with 0.6 M H\(_2\)O\(_2\) at 30°C and at pH 10, with BLA, which is the most thermostable natural α-amylase reported so far (29, 35), as control. AmyK38 retained full activity even over the course of 1 h, but the enzymatic activity of BLA rapidly decreased (half-life \[t_{1/2}\] = 3 min) in the presence of excess H\(_2\)O\(_2\). Moreover, AmyK38 maintained more than 80% of its original activity even after a 1-h incubation with 1.8 M H\(_2\)O\(_2\) (data not shown). These results indicate that AmyK38 is strongly resistant to chemical oxidation.

Effects of chelating reagents on the activity of AmyK38 were examined with BLA as control. AmyK38 and BLA were incubated with 1 mM EDTA at pH 10 in 50 mM glycine-NaOH buffer and at 40°C for up to 150 min. As shown in Fig. 5, both enzymes were stable in the absence of EDTA at least up to 150 min. In the presence of 1 mM EDTA, AmyK38 retained full activity even after incubation for 150 min, but BLA lost 88% of its original activity. Both enzymes were incubated with EDTA and EGTA at concentrations up to 100 mM in 50 mM glycine-NaOH buffer and at 40°C for up to 150 min. As control, the former (○) and the latter (□) enzymes were also incubated under the same conditions without EDTA. Samples (0.1 ml) were taken after the indicated times, and then the residual activity in the sample was immediately measured under the standard conditions of the enzyme assay. The values shown are percentages of the respective original activities, which are taken as 100%.

**FIG. 5.** Effect of EDTA on the activities of AmyK38 and BLA. AmyK38 (○) and BLA (□) (each at 2.0 U ml\(^{-1}\)) were each incubated with 1 mM EDTA at pH 10 in 50 mM glycine-NaOH buffer and at 40°C for up to 150 min. As control, the former (○) and the latter (□) enzymes were also incubated under the same conditions without EDTA. Samples (0.1 ml) were taken after the indicated times, and then the residual activity in the sample was immediately measured under the standard conditions of the enzyme assay. The values shown are percentages of the respective original activities, which are taken as 100%.

The primary goals for an optimally performing detergent α-amylase are high activity and stability in the temperature range from 40 to 60°C under alkaline pH conditions (7). Our alkaline α-amylase, AmyK38, characteristically shows high resistance to chemical oxidants and chelating reagents. Inactivation by chemical oxidation of an enzyme occurs mainly by oxidation of a methionine residue to its sulfoxide derivative. The oxidative inactivation hampers the industrial production and applications of enzymes and proteins. It is one of the most serious problems in the detergent industry because laundry and automatic dishwashing detergent formulations often contain bleach (1). To solve such problems, the oxidative stability...
of enzymes, subtilisins for example (9), has been improved by replacing oxidizable methionine with nonoxidizable amino acids using site-directed mutagenesis. However, we often encounter the reduction of catalytic activities of the improved mutant enzymes, including α-amylases. AmyK38 is highly resistant to chemical oxidation and exhibits high catalytic activity at alkaline pH compared with commercially available, neutral α-amylases such as BLA, BAA, and BSA. Moreover, our enzyme is very stable to incubation with chelating reagents, which are indispensable ingredients in detergent formulations. Therefore, our α-amylase is a high-performing enzyme even in detergent formulations. We are now cloning the gene for and resistance to chelating reagents and oxidative stability of the enzyme.

ACKNOWLEDGMENT

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