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Purification and Characterization of a *Bacillus* sp. DG0303 Thermostable α-Glucosidase with Oligo-1,6-glucosidase Activity

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Abstract Extracellular α-glucosidase was purified to homogeneity from moderately thermophilic *Bacillus* sp. DG0303. The thermostable α-glucosidase was purified by ammonium sulfate fractionation, ion-exchange chromatography, preparative polyacrylamide gel electrophoresis (PAGE), and electroelution. The molecular weight of the enzyme was estimated to be 60 kDa by SDS-PAGE. The optimum temperature for the action of the enzyme was at 60°C. It had a half-life of 35 min at 60°C. The enzyme was stable at the pH range of 4.5~7.0 and had an optimum pH at 5.0. The enzyme preparation did not require any metal ion for activity. The thermostable α-glucosidase hydrolyzed the α-1,6-linkages in isomaltose, isomaltotriose, and panose, and had little or no activity with maltooligosaccharides and other polysaccharides. The K_m (mM) for *p*-nitrophenyl-α-D-glucopyranoside (pNPG), panose, isomaltose, and isomaltotriose were 4.6, 4.7, 40.8, and 3.7 and the V_{max} (μmol · min^{-1} mg^{-1}) for those substrates were 5629, 1669, 3410, and 1827, respectively. The N-terminal amino acid sequence of the enzyme was MERVWWKKAV. Based on its substrate specificity and catalytic properties, the enzyme has been assigned to be an oligo-1,6-glucosidase.

Key words: *Bacillus* sp., thermostable, α-glucosidase, purification

α-Glucosidase covers a group of enzymes which hydrolyze terminal nonreducing α-D-glucosidic linkages of oligosaccharides and polysaccharides with the release of α-glucose. The substrate specificity of α-glucosidase differs greatly with the source of the enzyme [4]. The majority of α-glucosidases (α-D-glucoside glucohydrolase; EC 3.2.1.20) have been shown to preferentially hydrolyze maltose [9], whereas another class of α-glucosidases, oligo-1,6-glucosidases (dextrin 6-α-D-glucanohydrolase; EC 3.2.1.10), acts exclusively on the α-1,6-glucosidic linkage of isomaltooligosaccharides [13]. α-Glucosidase also catalyzes transglucosylation reactions when high concentrations of glucosyl acceptor are present in the reaction system [4]. It is considered as important an enzyme in the food industry as the isomaltooligosaccharides, which are synthesized by transglucosylation, have many properties usable in the food industry.

Many α-glucosidases have been reported to exist in microorganisms, animal tissues, and plants, and extensive studies have been done. Microbial α-glucosidases vary widely in their substrate specificity and molecular weight [4]. They have monomeric structures in general. However, oligomeric enzymes have been found in several thermophilic bacteria [8, 10, 16].

α-Glucosidase has been isolated from a variety of bacteria, including some moderate thermophiles. For example, Suzuki *et al.* [12] purified an extracellular α-glucosidase from *Bacillus thermoglucosidasius* which exhibited a temperature optimum of 75°C but was rapidly inactivated at higher temperatures. This enzyme has also been purified from other *Bacillus* species, including *Bacillus caldovelox* DSM411 [2] and *Bacillus stearothermophilus* ATCC12016 [14, 20]. These enzymes were shown to be optimally active at 60°C and 70°C, respectively. Purified α-glucosidase from a hyperthermophilic archaebacterium, *Pyrococcus furiosus*, exhibited a temperature optimum of 105°C to 115°C [1]. The thermostability of α-glucosidase has so far been analysed mainly in terms of amino acid compositions. Suzuki has proposed a general rule for protein thermostability, the 'proline theory', based on an observed strong correlation between thermostability and proline content of several *Bacillus* oligo-1,6-glucosidases [17]. Therefore, as a first step in studying the thermostability mechanism, we describe the purification and characterization of thermostable α-glucosidase from *Bacillus* sp. DG0303.

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MATERIALS AND METHODS

α-Glucosidase Assay

α-glucosidase activity was assayed spectrophotometrically by using *p*-nitrophenyl-α-D-glucopyranoside (pNPG) (Sigma, St. Louis, U.S.A.) as the substrate. The standard reaction mixture containing 1 ml of 0.1% pNPG, 1 ml of 150 mM sodium acetate buffer (pH 5.0), and 1 ml of enzyme solution was incubated at 60°C for 10 min, and the reaction was stopped by the addition of 2 ml of 1 M Na_2CO_3. The *p*-nitrophenol released was measured by the absorbance at 420 nm with a spectrophotometer (Shimadzu, model UV-160A, Kyoto, Japan). One unit of α-glucosidase activity was defined as the amount of enzyme that produced 1 μmole of *p*-nitrophenol per min under the conditions used.

Alternative substrates were used to determine the specificity of enzymatic action. Release of glucose was measured with a Sigma glucose diagnostic kit (Kit 510-A, colorimetric). Activity was expressed as μmoles of glucose liberated per minute. Protein was assayed with a protein assay kit (Bio Rad Laboratories, U.S.A.) using bovine serum albumin as the standard.

Purification of α-Glucosidase

Unless otherwise stated, all steps were conducted at room temperature. *Bacillus* sp. DG0303 was aerobically cultivated in LB medium at 55°C for 16 h. The culture supernatant obtained by centrifuging a 1 liter-culture was used as the crude enzyme source. Solid ammonium sulfate was added to the culture supernatant to 30% saturation and the mixture was left overnight at 4°C. The supernatant was collected by centrifugation and brought to 80% saturation in the same manner. The precipitate formed was collected by centrifugation, dissolved in 50 mM acetate buffer, pH 5.0, and dialyzed against the same buffer. The dialyzed enzyme solution was put on a CM-Sepharose column (2.5 × 15 cm) equilibrated with 50 mM acetate buffer (pH 5.0). The column was washed with the same buffer and absorbed proteins were eluted with 1 M NaCl in the same buffer. The enzyme activity was found in fractions eluted with the starting buffer. The enzyme solution passed through the CM-Sepharose column was applied to a Q-Sepharose column (2.5 × 25 cm) pre-equilibrated with 50 mM acetate buffer, pH 5.0. The column was washed extensively with the same buffer and then eluted with a linear gradient of 0 to 1 M NaCl in 50 mM acetate buffer (pH 5.0). The active α-glucosidase peak fractions were pooled, and concentrated with an Amicon cell equipped with a YM30 membrane. Preparative native polyacrylamide gel electrophoresis of the concentrated pool containing α-glucosidase activity from Q-Sepharose column and electroelution of α-glucosidase activity within the gel were performed as described below.

Electrophoresis and Electroelution

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was accomplished with the discontinuous system of Laemmli [6]. Protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue. Native polyacrylamide gel electrophoresis was conducted in the same manner as above except for the elimination of SDS. α-Glucosidase separated on native gel was localized by the two-step activity staining method of Spielman and Mowshowitz [11]. The activity band was cut from the gel, and electroelution was accomplished with Elutrap equipment (Schleicher & Schuell Inc., U.S.A). For electroelution, the running buffer was the same as that used for native polyacrylamide gel electrophoresis. Following electroelution, the sample was dialyzed against 50 mM sodium acetate (pH 5.0).

Effects of pH and Temperature on α-Glucosidase Activity

The relative activity of the enzyme was determined at different pH values by using 50 mM McIlvaine (citric acid-Na_2HPO_4) buffer for the range from pH 3 to 7.5, and sodium phosphate buffer for the range from pH 6 to 8.5. The temperature dependence of the enzyme was determined by measuring the relative enzyme activity at temperatures between 30 and 80°C. To determine thermostability of α-glucosidase, purified enzyme was incubated in microcentrifuge tubes in water baths for various lengths of time. The remaining α-glucosidase activity was measured as described above.

Substrate Specificity

The enzyme reaction was carried out as described for enzyme assay, except that the reaction mixture contained 10 mM or 0.1% of various substrates. The reaction was stopped by heating the mixture for 3 min at 95°C and the mixture was assayed for glucose. Kinetic parameters of glucose release from substrates were determined under the above assay conditions and were expressed as micromoles of reducing groups per min per milligram protein, with glucose as the standard. K_m and V_{max} values were obtained from Lineweaver-Burk plots.

Effects of Metal Ions

To test the inhibition by various metal ions and EDTA, the purified enzyme was preincubated at 55°C for 30 min in 50 mM acetate buffer (pH 5.0) containing each reagent. Substrate was then added and the enzyme activity was measured as described above.

N-Terminal Amino Acid Sequencing

The purified enzyme was subjected to SDS-PAGE. Electrotransfer of proteins to polyvinylidene difluoride (PVDF) membrane was carried out according to the

Table 1. Purification of the α-glucosidase from *Bacillus* sp. DG0303.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Culture supernatant	5040.6	57657	11	100
Ammonium sulfate	113.5	39702	350	69
CM-Sepharose	25.1	26908	1072	46.6
Q-Sepharose	3.0	17933	5978	31.1
Electroelution	0.08	3777	47213	6.6

method described previously [7]. Analysis of the N-terminal sequence was carried out by automated Edman degradation.

RESULTS

Purification of α-Glucosidase

The purification procedure is shown in Table 1. The final enzyme preparation was purified 4292-fold over the activity in the starting culture supernatant, with a final yield of 6.6%

Hydrophobic chromatography with phenyl-Sepharose, hydroxyapatite chromatography, and gel filtration chromatography were tried but they did not increase the specific activity. The elution profile from a Q-Sepharose column showed that one active peak was eluted over 0.30~0.35 M NaCl (Fig. 1). A specific staining procedure has been developed for the identification of α-glucosidases in polyacrylamide gels [11]. This method is sensitive and does not inactivate the enzyme activity. SDS-polyacrylamide gel electrophoresis of the purified α-glucosidase is shown in Fig. 2. Coomassie Blue staining revealed one band having a molecular mass of about 60 kDa. This value is in good agreement with the molecular mass determined by gel-filtration chromatography and suggests that the native enzyme is a monomer. Gel activity staining confirmed that this band was responsible for the α-glucosidase activity.

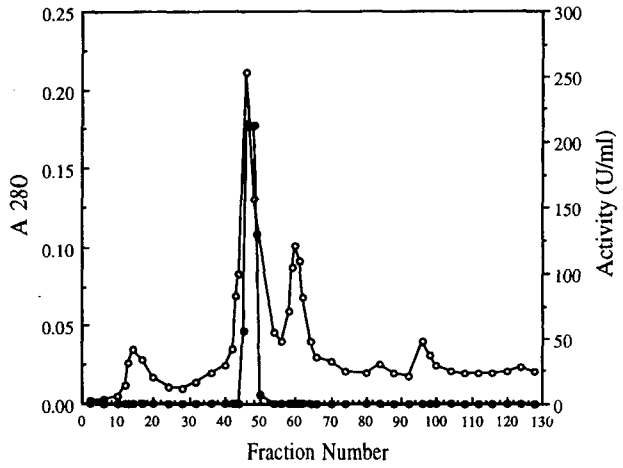

Fig. 1. Elution profile of α-glucosidase activity (●) and of protein (○) from a Q-Sepharose column.

The enzyme solution passed through the CM-Sepharose column was applied to the Q-Sepharose column and eluted as described in Materials and Methods. The activity is expressed in U/ml eluate. The A280 of each fraction was measured as an indication of protein content.

Effects of pH and Temperature on α-Glucosidase Activity

The effect of pH on α-glucosidase activity was determined at 55°C (Fig. 3). At this temperature, the α-glucosidase exhibited optimum activity at pH 5.0 and the enzyme remained stable between pH 4.5 and 7 for at least 30 min. This characteristic is typical of many α-glucosidases [4]. Figure 4 shows the effect of assay temperature on the α-glucosidase activity. The optimum

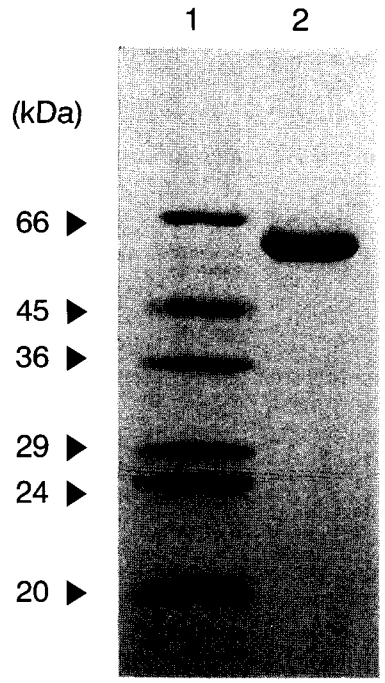

Fig. 2. SDS-polyacrylamide gel electrophoresis of *Bacillus* sp. DG0303 α-glucosidase.

Purified enzyme was run in lane 2. Lane 1 contains protein molecular size standards (top to bottom): bovine albumin (66 kDa), egg albumin (45 kDa), G-3-P dehydrolase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20 kDa). SDS-PAGE and protein staining were performed as described in Materials and Methods.

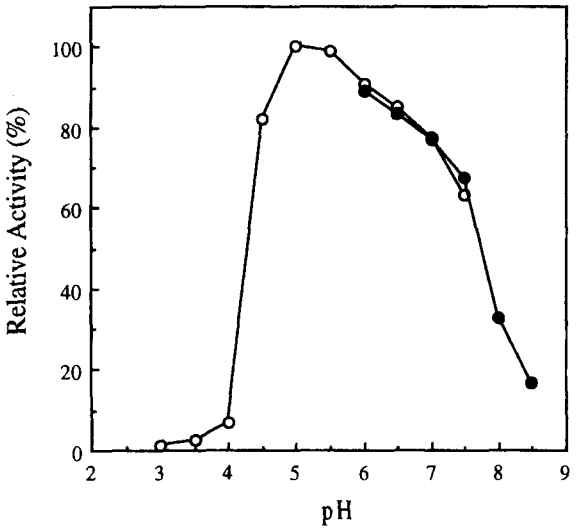

Fig. 3. Effect of pH on the α-glucosidase activity.
The relative α-glucosidase activity of the purified enzyme was determined at various pHs. The buffers used were McIlvaine buffer (○) and sodium phosphate buffer (●).

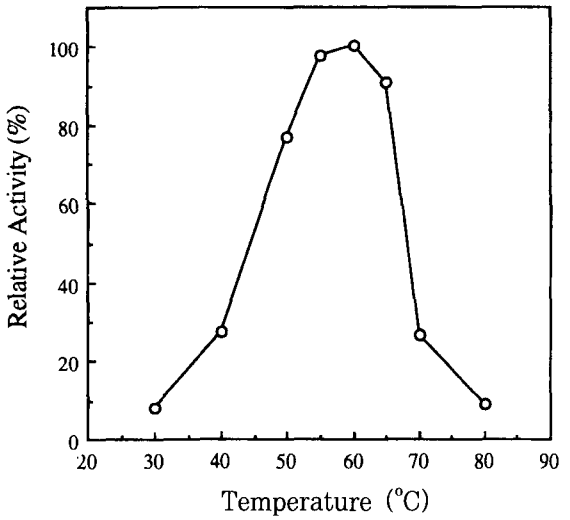

Fig. 4. Effect of temperature on the α-glucosidase activity.
The relative α-glucosidase activity of the purified enzyme was determined at various temperature. The reaction was done in 50 mM sodium acetate buffer (pH 5.0).

temperature for catalytic activity was 60°C. In many instances, bacterial α-glucosidases have pH optima near 7.0 or in the acidic region and temperature optima near 40°C [3, 21, 22, 23].

Thermostability of α-Glucosidase

Portions of purified α-glucosidase incubated at 55°C, 60°C, and 65°C, pH 5.0, were taken at various time points, and the remaining activity was determined (Fig. 5). There was no loss of activity after 6 h of incubation at 55°C. More than 80% loss of activity was observed

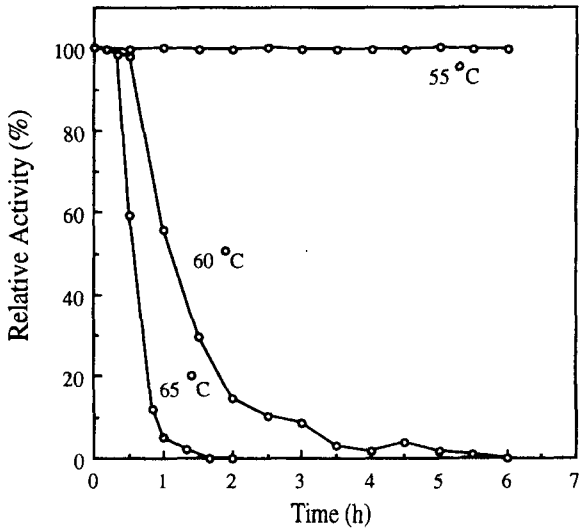

Fig. 5. Thermostability of *Bacillus* sp. DG0303 α-glucosidase.
Purified enzyme in acetate buffer (50 mM, pH 5.0) was incubated at various temperatures and residual activities at various time intervals were assayed by the standard assay method.

Table 2. Relative levels of inhibition of extracellular α-glucosidase from *Bacillus* sp. DG0303 by metal ions.

Compound	% of residual activity at final concentration of	
	2 mM	10 mM
None	100	100
$ZnCl_2$	77	43
$AgNO_3$	0	0
$CuSO_4$	29	0
$CoCl_2$	97	90
$CaCl_2$	96	91
$MgSO_4$	102	106
$MnCl_2$	101	94
$FeSO_4$	2	1
$HgCl_2$	1	0
EDTA	108	108

after 2 h of incubation at 60°C. This enzyme exhibited moderate thermostability, with a half-life of about 35 min at 65°C. The thermostability of the enzyme was drastically decreased above 70°C.

Effects of Metal Ions

Inhibition by metal ions was studied by incubating the enzyme in the presence of 2 or 10 mM metal ions (Table 2). The purified enzyme was inhibited by Zn^{2+}, Ag^{+}, Cu^{2+}, Fe^{2+}, and Hg^{2+} ions when the final concentratons were 2 mM. Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+} ions had no effect on the enzyme activity (relative activity, >90%). Thus, this enzyme does not require any metal ion for activity. EDTA at 10 mM concentration did not inhibit the α-glucosidase activity.

Table 3. Substrate specificity of purified α-glucosidase from *Bacillus* sp. DG0303.

Substrate[a] (10 mM)	Relative hydrolysis (%)[b]
p-Nitrophenyl-α-D-glucoside	100
Isomaltose	24
Isomaltotriose	60
Panose	54
Maltose	5
Maltotriose	4
Maltotetrose	3
Methyl-α-D-glucoside	3

[a]Obtained from Sigma (U.S.A.).
[b]Determined after 10 min at 60°C and pH 5.0.

Substrate Specificity

The substrate specificity of the purified enzyme was examined on various glucosides at 60°C (Table 3). The purified enzyme had greatest preference for *p*-nitrophenyl-α-D-glucopyranoside (pNPG). Analysis of the activity of the enzyme toward a range of substrates indicated a preference for short isomaltosaccharides and panose. The enzyme had little or no activity against maltose, maltotriose, maltotetrose, sucrose, methyl α-D-glucoside, *p*-nitrophenyl-α-D-xylopyranoside, cellobiose (each 10 mM), soluble starch, pullulan, dextrin, dextran, or β-cyclodextrin (each 1%). No activity was observed against the β-linked glucosides such as *p*-nitrophenyl-β-D-glucopyranoside, indicating enzyme preference for α-linked glucosides. The α-glucosidase displayed the ability to hydrolyse α-1,6-linkage, but showed low or no activity on α-1,4-linkages, or on alkyl-α-D-glucosides.

Kinetic Analysis of α-Glucosidase

To characterize the substrate specificity of the enzyme more closely, we measured the saturation kinetics of glucose release from different substrates (Table 4). The enzyme showed greater affinity for isomaltotriose than for isomaltose, but the maximum velocity of glucose release was about twofold higher for isomaltose. For panose, affinity was slightly lower than isomaltotriose, but hydrolysis was comparable to the rate for isomaltotriose. We also determined K_m and V_{max} values for pNPG, the synthetic chromogenic substrate for α-glucosidase. The binding of the synthetic substrate was similar to those of panose and isomaltotriose, but the maximum velocity was about threefold higher than these substrates.

Table 4. Kinetic parameters of α-glucosidase activity.

Substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
pNPG	4.6	5629
Panose	4.7	1669
Isomaltose	40.8	3410
Isomaltotriose	3.7	1827

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                1               5              10
Bsp1    Met-Glu-Arg-Val-Trp-Trp-Lys-Lys-Ala-Val-
Btg     Met-Glu-Arg-Val-Trp-Trp-Lys-Glu-Ala-Val-
Bta1    Met-Glu-Arg-Ala-Trp-Trp-Lys-Glu-Ala-Val-
Bce     Met-Glu-Lys-Gln-Trp-Trp-Lys-Glu-Ser-Val-
Bst     Met-Lys-Lys-Thr-Trp-Trp-Lys-Glu-Gly-Val-
Bta2    Met-Lys-Lys-Ala-Trp-Trp-Lys-Glu-Gly-Val-
Bsp2    Ile-Arg-Arg-Ala-Trp-Trp-Lys-Glu-Ala-Val-
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Fig. 6. N-Terminal sequences of *Bacillus* sp. DG0303 α-glucosidase (Bsp1), *B. thermoglucosidasius* oligo-1,6-glucosidase (Btg) [24], *B. thermoamyloliquefaciens* oligo-1,6-glucosidase (Bta1) [19], *B. cereus* oligo-1,6-glucosidase (Bce) [25], *B. stearothermophilus* α-glucosidase (Bst) [20], *B. thermoamyloliquefaciens* α-glucosidase I (Bta2) [19] and *Bacillus* sp. SAM1606 α-glucosidase (Bsp2) [8].

N-Terminal Amino Acid Sequence Analysis

The N-terminal amino acid sequence of purified enzyme was determined and compared with those of other α-glucosidases (Fig. 6). The N-terminal amino acid sequence of DG0303 α-glucosidase was determined as MERVWWKKAV. This showed a very high sequence identity to the N-terminal amino acid sequence of oligo-1, 6-glucosidase of *Bacillus thermoglucosidasius* KP1006, *B. thermoamyloliquefaciens* KP1071, and *B. cereus* ATCC 7064. Sequence similarity was also found with α-glucosidase from *B. stearothermophilus, B. thermoamyloliquefaciens*, and *Bacillus* sp. SAM1606.

DISCUSSION

Microbial α-glucosidases exhibit significant diversity in their glucoside substrate specificity. Additionally, these enzymes can be either intracellular, extracellular, or membrane bound. Thus, classification and enzymatic comparisons among α-glucosidases are difficult. Recently, Kelly and Fogarty [5] proposed a reclassification of bacterial α-glucosidase enzymes into those with highest specific activity toward maltose and those with highest specific activity toward aryl-D-glucosides.

In general, bacterial α-glucosidases are only moderately thermostable [4]. For example, the extracellular α-glucosidase from *Bacillus thermoglucosidasius* KP 1006 [12], although stable at 60°C for 2 h with no loss of activity, was highly unstable at temperatures above 72°C. At temperatures above this, the enzyme exhibited a half-life of only 10 min or less. Similarly, the exo-α-1,4-glucosidase from *B. stearothermophilus* ATCC12016 [14], which was optimally active at 70°C, retained only 7% of initial activity when incubated for 10 min at this temperature. The α-glucosidase from *Pyrococcus furiosus* [1] demonstrated the greatest thermostability. This enzyme demonstrated a half-life of about 48 h at 98°C.

The α-glucosidase from *Bacillus* sp. DG0303 greatly prefers α-1,6 bonds to α-1,4 bonds (Table 3). The purified α-glucosidase hydrolyzes α-1,6-glucosidic linkages in exo-fashion from nonreducing termini in short isomaltooligosaccharides and panose, but fails to act on the same linkages in the corresponding polymers, dextran and pullulan. This is a distinct catalytic property of *Bacillus* oligo-1,6-glucosidases [16]. This enzyme also cannot act on either α-1,4 or α-1,6 bonds in β-limit dextrin. This restricted substrate specificity and the kinetic measurements indicate that the enzyme is an exo-oligo-1,6-glucosidase. α-Glucosidase from *Bacillus* sp. DG0303 surprisingly resemble oligo-1,6-glucosidase from *Bacillus thermoglucosidasius, B. thermoamyloliquefaciens*, and *B. cereus* in molecular weight, substrate specificity, and in the N-terminal sequence of 10 residues. Therefore, *Bacillus* sp. DG0303 α-glucosidase can be assigned to an oligo-1,6-glucosidase. Oligo-1,6-glucosidases (dextrin 6-α-D-glucanohydrolase, EC 3.2.1.10) has been isolated from a variety of bacteria, including some moderate thermophiles. For example, Suzuki *et al.* [12] purified an extracellular oligo-1,6-glucosidase from *Bacillus thermoglucosidasius* KP1006 which exhibited a temperature optimum of 75°C. This enzyme has also been purified from other *Bacillus* species, including *Bacillus cereus* ATCC7064, *Bacillus coagulans* ATCC7050, and *Bacillus flavocaldarius* KP1228 [13, 15, 16].

Suzuki *et al.* [13, 15, 16, 17, 18] found a strong correlation between the increase in the number of proline residues and the rise in the thermostability of bacillary oligo-1,6-glucosidases. On the basis of this finding, Suzuki *et al.* [17] proposed an idea (the proline rule) which stated that the increases in the frequency of proline occurrence at β turns and in the total number of hydrophobic residues can enhance protein thermostability. We are now in the progress of cloning and sequencing the structural gene encoding the oligo-1,6-glucosidase from *Bacillus* sp. DG0303. We hope that this investigation will provide more information on the relationship between the proline content and protein thermostability.

Acknowledgments

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