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New alkalophilic β-galactosidase with high activity in alkaline pH region from *Teratosphaeria acidotherma* AIU BGA-1

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A β-D-galactosidase exhibiting high activity in the alkaline pH region was purified from *Teratosphaeria acidotherma* AIU BGA-1, which we previously isolated as a unique fungal producer of three acidophilic and one alkalophilic β-D-galactosidases (Isobe et al., J. Biosci. Bioeng., 116, 171–174, 2013). The enzyme was stable in the pH range 7.5–10.0 and exhibited optimal activity at pH 8.0 and 60°C. The enzyme hydrolyzed 2-nitrophenyl β-D-galactopyranoside, 4-nitrophenyl β-D-galactopyranoside, and lactose, and the K_m values were estimated to be 0.349 mM, 0.488 mM, and 701 mM, respectively. Chelating reagents (EDTA and *o*-phenanthroline) and metals (Cu^{2+} and Ni^{2+}) inhibited the enzyme activity, and Mn^{2+} was a good activator. The enzyme also exhibited transgalactosylation activity for lactose. The enzyme's molecular mass was estimated to be 180 kDa, and its structure was monomeric. Thus, the enzymatic and physicochemical characteristics of the alkalophilic β-galactosidase in this study clearly differed from those of the previously known alkalophilic β-D-galactosidases.

[**Key words:** β-Galactosidase; Lactase; Alkalophilic lactase; *Teratosphaeria acidotherma*; Lactose]

β-D-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), commonly known as lactase can catalyze both hydrolysis and transgalactosylation in the presence of lactose. The hydrolytic potential of β-D-galactosidase is widely utilized in the dairy industry to improve the digestibility, solubility, or sweetness of lactose, and to produce lactose-free milk for lactose-intolerant people (1). The transgalactosylation potential is utilized in the production of prebiotic galacto-oligosaccharides (GOS) and in the modification of structural and functional characteristics of food materials or pharmaceutical compounds (1). Thus, β-D-galactosidase is one of the most important enzymes in the dairy, food, and pharmaceutical industries.

Many β-D-galactosidases with various optimal pH values have been found from microorganisms. Enzymes exhibiting high activity in the neutral pH region were widely obtained from yeasts (2–5) and bacteria (6–10), and those exhibiting high activity in the acidic pH region were from fungal strains (11–14). However, there are few reports on alkalophilic β-D-galactosidases whose highest activity is in the pH range 8.0–10.5. In addition, all alkalophilic β-D-galactosidases reported previously were produced by bacteria; enzymes from *Meiothermus ruber* DMS 1279 (15) and *Bacillus* sp. MTCC 3088 (16) showed high activity at high temperatures (65°C and 60°C, respectively, at pH 8.0), and those from psychrophilic bacteria such as *Pseudoalteromonas* sp. (17), *Arthrobacter* sp. ON14 (18), and *Arthrobacter psychrolactophilus* strain F2 (19) showed high activity at low temperatures (26°C at pH 9.0, 15°C at pH 8.0, and 10°C at pH 8.0, respectively). The enzyme from *Enterobacter cloacae* B5 exhibited high activity at pH 7.5–10.5 and 35°C (20). In these alkalophilic β-D-galactosidases, the enzymes from *M. ruber* DMS 1279 (15), *Bacillus* sp. MTCC 3088 (16), and *E. cloacae* B5 (20) were investigated for their potential use in the biocatalytic production of GOS.

Recently, we isolated *Teratosphaeria acidotherma* AIU BGA-1 as a new producer of β-D-galactosidases, and revealed that this strain produced three acidophilic β-D-galactosidases with different pH activity profiles as well as one alkalophilic β-D-galactosidase (21). Then, we purified the two acidophilic β-D-galactosidases [P-2 and P-4 enzymes in our previous report (21)] from the strain. The P-2 enzyme exhibited its highest activity at pH 1.0, which is the lowest optimal pH value among the microbial β-D-galactosidases (14). The P-4 enzyme was stable in a wide pH range from extremely acidic to neutral, and exhibited high activity from pH 1.0 to pH 7.0 (13). These enzymatic properties of the β-D-galactosidases from *T. acidotherma* AIU BGA-1 are more advantageous for industrial application than those of the other β-D-galactosidases.

In the present study, we purified the alkalophilic β-D-galactosidase from the same strain [P-1 enzyme in our previous report (21)] and revealed its certain remarkable characteristics relative to those of other microbial β-D-galactosidases.

MATERIALS AND METHODS

Chemicals Lactose and 2-nitrophenyl β-D-galactopyranoside (2-NPGA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-Nitrophenyl β-D-galactopyranoside (4-NPGA), 4-nitrophenyl β-D-glucopyranoside (4-NPGL), 4-nitrophenyl β-D-xylopyranoside (4-NPXY), 4-nitrophenyl β-D-fucopyranoside (4-NPFU), 4-nitrophenyl β-D-mannopyranoside (4-NPMA), and 4-nitrophenyl β-L-arabinofuranoside (4-NPAR) were purchased from Sigma–Aldrich (St. Louis, MO,

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USA). Peroxidase and glucose oxidase were gifts from Amano Enzyme (Nagoya, Japan). All other chemicals were of analytical grade and commercially available.

Cultivation of the strain *T. acidotherma* AIU BGA-1 was incubated with lactose medium, pH 5.0, consisting of 2.0% lactose, 0.2% corn steep liquor, 0.2% KH_4NO_3, 0.1% KH_2PO_4, 0.1% Na_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.15%$Ca(NO_3)_2 \cdot 4H_2O$, and 0.1% $FeCl_3 \cdot 6H_2O$, at 30°C for 2 days according to our previous report (14).

Assay of enzyme activity The alkalophilic β-D-galactosidase activity was assayed at pH 8.5 using 12.5 mM 2-NPGA dissolved with 0.1 M NH_4Cl–NH_3, pH 8.5, as follows. A reaction mixture containing 0.2 ml of 12.5 mM 2-NPGA, pH 8.5, and 0.05 ml of enzyme solution was incubated at 37°C for 15 min. The reaction was terminated by adding 0.25 ml of 10% Na_2CO_3, and the amount of 2-nitrophenol released was determined by measuring the absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the release of 1 μmol of 2-nitrophenol per min under the above conditions. The molar absorptivity of the dye formed under the above conditions was $4.6 \times 10^3\ M^{-1}\ cm^{-1}$.

The lactose-hydrolyzing activity of β-D-galactosidase was assayed at pH 8.5 using the glucose oxidase method described in our previous report (13).

Purification of the enzyme All the procedures were performed at 5–10°C using potassium phosphate buffer. The mycelia from 12 L of culture broth (120 g wet weight) were disrupted with glass beads in 10 mM buffer solution, pH 7.0, by a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan), and the supernatant (2.5 L) collected by centrifugation at 10,000 ×*g* for 10 min was used as a crude enzyme solution. The enzyme solution was then applied onto a DEAE-Toyopearl 650M column (46 × 2.5 cm diameter; Tosoh Bioscience, Tokyo, Japan) equilibrated with 20 mM buffer solution, pH 6.7. The adsorbed enzyme was eluted by a linear gradient with 20 mM buffer solution and 20 mM buffer solution containing 100 mM NaCl (1 L each). The active fractions were collected, and solid ammonium sulfate was added up to 1.2 M. The enzyme solution was applied to a Phenyl-Toyopearl 650M column (23 × 2.5 cm diameter; Tosoh Bioscience) equilibrated with 10 mM buffer solution containing 1.2 M ammonium sulfate, pH 7.5. The adsorbed enzyme was eluted by a linear gradient with 10 mM buffer solution containing 1.2 M and 0.4 M ammonium sulfate, pH 7.5 (500 ml each). The active fractions were collected and dialyzed against 10 mM buffer solution, pH 7.0. Next, the dialyzed enzyme was applied to a hydroxyapatite column (18 × 1.8 cm diameter) equilibrated with 20 mM buffer solution, pH 7.5, and the adsorbed enzyme was eluted by a linear gradient with 10 mM and 90 mM buffer solution, pH 7.5 (100 ml each). The active fractions were combined and used for the following studies.

SDS-PAGE and molecular mass measurement The sample for SDS-PAGE was incubated with 1% SDS and 5% mercaptoethanol at 100°C for 3 min. SDS-PAGE was performed according to the method of Laemmli (22). Proteins were stained with Coomassie Brilliant Blue R-250. The molecular mass of the denatured enzyme was estimated by SDS-PAGE using the molecular marker standards of Bio-Rad Japan (Tokyo, Japan). The molecular mass of the intact protein was estimated by gel filtration on a TSK gel G3000SW_{XL} column (Tosoh Bioscience). The protein concentration was spectrophotometrically determined by measuring absorbance at 280 nm. An $E^{1\%}_{1\ cm}$ value of 10.0 was used throughout this work.

Hydrolysis of lactose and TLC analysis The enzyme (16 mU) was incubated with 300 mM lactose and 2 mM $MnCl_2$ at 37°C for 20 h at pH 8.5 (reaction volume was 100 μl), and the reaction was terminated by boiling for 3 min. TLC analysis of the reaction products from lactose was carried out using a silica gel plate (Silica Gel 60F 254; Merck, Germany) and a solvent composed of *n*-butanol:*n*-propanol:ethanol:water (2:3:3:2). The spots were detected by heating for a few minutes on a hot plate after spraying with a solution containing 20 mg of diphenylamine, 20 μl of aniline, and 0.1 ml of phosphoric acid in 1.0 ml of acetone.

RESULTS

Purification and molecular mass of the enzyme The alkalophilic β-D-galactosidase was purified from mycelia of *T. acidotherma* AIU BGA-1 to an electrophoretically homogeneous state by means of three column chromatographies (Table 1). Finally, an approximately 3400-fold purification was achieved with an overall yield of 11.9%. The purified enzyme showed a single protein band on native- and SDS-PAGE, and the molecular mass of the denatured enzyme was estimated to be 120 kDa (Fig. 1). The molecular mass of the native enzyme was estimated to be approximately 180 kDa on a TSK gel G3000SW_{XL} column (Tosoh Bioscience) (data not shown). These results indicated that the alkalophilic β-D-galactosidase from *T. acidotherma* is a monomeric enzyme.

TABLE 1. Purification of alkaline β-D-galactosidase from *T. acidotherma* AIU BGA-1.

Step	Activity (units)	Protein (mg)	Specific activity (unit/mg protein)	Recovery (%)	Purification (fold)
Cell-free extract	54.6	45800	0.00119	100	1.00
DEAE-Toyopearl	12.4	89.6	0.138	22.7	116
Phenyl-Toyopearl	10.8	3.1	3.48	19.8	2920
Hydroxylapatite	6.48	1.6	4.05	11.9	3400

Enzyme activity was assayed under standard assay conditions at pH 8.5. Specific activity was expressed as units per milligram of protein.

Substrate specificity and kinetic parameters The enzyme exhibited highest activity for 4-NPGA, and the activity for 2-NPGA was 38% of that for 4-NPGA. However, the enzyme did not hydrolyze 4-NPGL, 4-NPXY, 4-NPFU, 4-NPMA, or 4-NPAR (Table 2). The K_m and V_{max} values for 2-NPGA were estimated to be 0.35 mM and 4.89 μmol/min/mg protein and those for 4-NPGA were estimated to be 0.49 mM and 14.7 μmol/min/mg protein (Table 3). The enzyme also hydrolyzed lactose, whose K_m and V_{max} values were estimated to be 701 mM and 0.38 μmol/min/mg protein by the glucose oxidase method. These results indicated that the enzyme efficiently hydrolyzed a high concentration of lactose.

Effects of compounds on enzyme activity The effects of various compounds on β-D-galactosidase activity were analyzed with the addition of 1 mM carbonyl reagents, chelating reagents, metals, or other chemicals (Table 4). The enzyme activity was inhibited strongly by EDTA and hydrazine, and slightly by hydroxylamine, *o*-phenanthroline, sodium azide, and *N*-ethylmaleimide among the

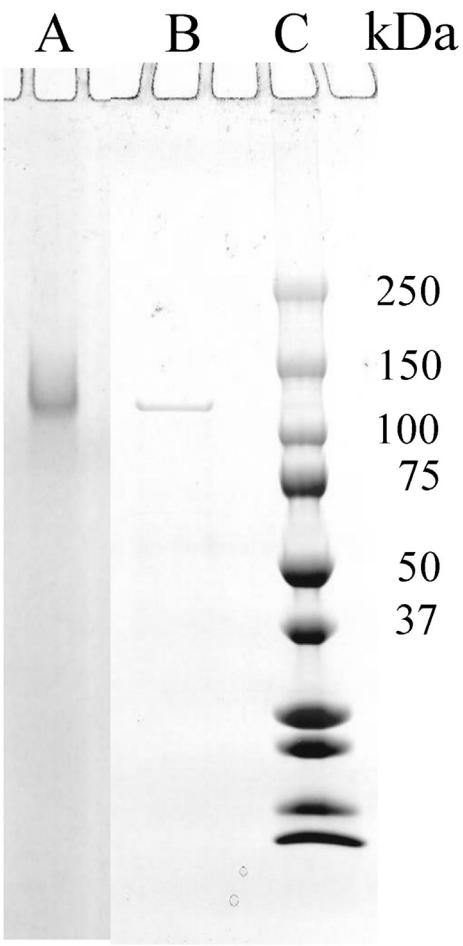

FIG. 1. Native- and SDS-PAGE of purified alkaline β-D-galactosidase from *T. acidotherma* AIU BGA-1. (A) Native enzyme, (B) denatured enzyme, (C) molecular markers.