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Regular Article

Assignment of KP1246, a thermophilic actinomycete strain that produces 2 distinct β-glucosidases, to *Thermomonospora curvata*

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From the culture supernatant of a thermophilic actinomycete KP1246, which grows at 45°C to 66°C, two electrophoretically homogenous *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG)-degrading β-glucosidases (BGL1, BGL2) were obtained by the combination of an ion-exchange and gel-filtration chromatography. The molecular weight, Stokes radius, and sedimentation coefficient of BGL1 were found to be 51,000, 2.92, and 4.4S, respectively, which were similar to those of BGL2 (50,000, 3.06, and 4.2S, respectively). However, differences were observed in the isoelectric point (pI) (5.4 for BGL1 and 4.2 for BGL2) and in the optimum pH for activity (6.6 for BGL1 and 5.5 for BGL2). The optimum temperature for activity was found to be 70°C for BGL1 and 55°C for BGL2. In the study on the retention of heat resistance after 30 min of processing, BGL1 was demonstrated to retain 100% of

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the original activity over a wide range of pH, from 5.0 to 11.0. Meanwhile, the stability range of pH was found to be narrower, i.e., 7 to 9.5, for BGL2. For both BGL1 and BGL2, *p*NPG, as well as cellobiose and salicin, were found to be good substrates. Neither BGL1 nor BGL2 reacted with Avicel, carboxymethylcellulose (CM-cellulose), or maltose. Enzymatic reaction of BGL1 was not at all inhibited by glucose; however, in the enzymatic reaction of BGL2, glucose acted as a competitive inhibitor (*Ki* = 408 mM). Cellobiose acted as a noncompetitive inhibitor to BGL1 (*Ki* = 50 mM) and showed a similar inhibition format to BGL2 (*Ki* = 15.6 mM). Addition of 2 mM EDTA, Pb^{2+}, Fe^{2+}, Mn^{2+}, or Mg^{2+} did not at all inhibit the activity of BGL1; however, these resulted in 26.1%, 25.2%, 22.3%, 17.5%, and 10.5% inhibition of BGL2 activity, respectively. *p*CMB (*p*-chloromercuribennzoate) was found to inhibit BGL1 activity by 100%; however, the level of inhibition was only 32.5% for BGL2. The results of this kinetic study, which is the first of its kind, on the thermophilic actinomycete *T. curvata* suggest that at least 2 different types of heat-resistant BGLs function in the final stage of the cellulase degradation system of the KP1246 strain and that the strain can be classified as *T. curvata*.

Key words: *Thermomonospora curvata*; *Thermobifida fusca*; glycoside hydrolase family; β-glucosidase; cellobiose

Introduction

In recent years, there is a concern for the depletion of fossil fuels, and there is much expectation for the technology of converting plant biomass into bioethanol. Cellulose is the predominant component of plant biomass and as such is the most abundant organic polymer on earth (1-3). *Thermomonospora* sp., a thermophilic actinomycete can grow at 50 – 60°C in defined medium and thus is a major degrader of cellulose materials in heated organic materi-

als such as compost piles (4-6). The enzymatic hydrolysis by *Thermomonospora* sp. for the conversion of cellulose to glucose, involves synergistic activity of three types of cellulases (7 - 11): (i) endoglucanases (EG, EC3.2.1.4), which hydrolyze bonds internally in cellulose chains (12,13), (ii) cellobiohydrolases (CBH, EC3.2.1.91), which act preferentially on chain ends and progressively cleave off cellobiose as the main product (14,15), and (iii) β-glucosidase (BGL, EC3.2.1.21), also known as β-D-glucoside glucohydrolase that is positioned at the terminal phase and acts as a catalyst in the hydrolysis of the β-glycoside binding in glucose (4, 16). Therefore, β-glucosidase becomes important in this process, since cellulose needs to be hydrolyzed completely to produce glucose. Although, kinetic modeling of detailed data dealing with *Themomonospora* sp. were reported for both EG and CBH (17-20) , there has not been well characterized for BGL, possibly because of the difficulty of purifying BGL from this monosporic thermophile.

Strain of *T. curvata* KP1246 was found to secrete both EG and CBH and the final product of KP1246 cellulase system was glucose (22, 23). In the present study, we have focused on detection and purification of two types of BGLs in *T. curvata*, and for the first time aimed to present the kinetic parameters of thermophilic actinomycete BGLs.

Materials and methods

Enzyme assay. BGL activity was determined photometrically at 55℃ in a reaction mixture (1.0 ml) containing 30 mM potassium phosphate buffer (PPB, pH 6.8), 2 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and enzyme (21, 22). One unit (U) of enzyme activity was defined as the amount of enzyme hydrolyzing 1.0 µmol of nitrophenyl glucoside/min under the above conditions. When various sugars were employed as substrates, either reducing power or glucose formed was

assayed by using dinitrosalicylic acid (23, 24) and Glucose C test kit (Wako Chemicals: 25), respectively. One unit (U) of enzyme activity was defined as the amount of enzyme reducing 1.0 μmol sugars formed/ min. Protein was assayed according to the method of Lowry et al using bovine serum albumin as the standard (26).

Microbial strains and culture conditions. Stock culture of *T.curvata* (21) was subjected to pre-incubation at 58℃ for 48 h on five medium I agar (3.0%, w/v) plates, consisting of 1.0% meat extract, 1.0% peptone, 0.5% NaCl (pH6.8). Cells collected from the plates were suspended in a 2-liter Erlenmeyer flask, containing of 200 ml medium II (pH 6.8), which contained 0.3 % K_2HPO_4 , 0.1% KH_2PO_4, 0.3% peptone, 0.15% yeast extract, 0.002% $MgSO_4 \cdot 7H_2O$, 0.005% FeSO4 · $7H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$, 0.002% $MnCl_2 \cdot 4H_2O$, and 0.001% $NaMoO_4 \cdot 2H_2O$ and shaken at 58℃ for 2 h with twenty pieces of glass beads (3 mm in diameter) and at 198 cycles/min (3.4 cm amplitude) with a rotary shaker. The turbid cultures after filtration through three layers of cheese cloth were diluted with 0.85% NaCl to give an absorbance of 0.50- 0.65 at 660 nm (1.8 cm light path). The ten-ml aliquots of cell suspensions obtained above were inoculated to twenty 2-liter Erlenmeyer flasks, containing 200 ml of medium II, supplemented with 0.5% cellulose powder (Nakalai Tesque, Kyoto, Japan), and shaken at 58℃ for 5 days.

Purification of enzyme. In the following procedures, all operations were conducted at 4°C unless otherwise specified.

Step1 Culture supernatant. The culture solution after 8 days of incubation was filtered with Toyo filter paper No. 1. The culture supernatant obtained (25 L) was concentrated at 30°C under reduced pressure with a rotary evaporator. The concentrated supernatant was then centrifuged for 30 min at 10,000

rpm at 4°C to obtain a clear culture supernatant (volume: 1.7 L).

Step2 Ammonium sulfate fractionation. Solid ammonium sulfate was added to the culture supernatant so that the concentration would be 40% saturation. This was stirred for 30 min, then left standing for 1 h. To the supernatant obtained after centrifugation, ammonium sulfate was added so that the final concentration would be 65% saturation; then, this was left standing. After centrifugation, the precipitate obtained was dissolved in 5 mM PPB, and centrifuged again under the same conditions to obtain 163 mL of clear solution.

Step3 Acetone fractionation. To the solution obtained in Step 2, acetone stored at minus 30°C was added over 10 min while stirring, so that the final concentration would be 50% saturation. This solution was left standing for 30 min and then centrifuged. Acetone was added to the supernatant obtained so that the final concentration was 200% saturation. This was left standing and then centrifuged. The precipitate was air-dried for 3 min in a desiccator and dissolved in 5 mM PPB (pH 6.8); then, centrifugation was performed and supernatant (volume: 88 mL) was obtained.

Step4 Sephadex G-75 gel column chromatography. Of the total volume of the supernatant obtained in Step 3, 1/2 (44 mL) was applied to Sephadex G-75 column (4.4 × 96.8 cm), which was equilibrated with 20 mM PPB (pH 6.8)/0.5M NaCl/0.02% sodium azide beforehand. This was fractionated by 15 mL into each tube at a flow rate of 75 mL/h to obtain the enzyme activity fractions (tube numbers 36–51). The same procedures were performed for the remaining supernatant (44 mL), and the two activity fractions were combined (volume: 330 mL).

Step5 DEAE-Sepharose ion-exchange chromatography. The solution obtained in Step 4 was dialyzed and applied to the DE-AE-Sephadex A-50 column (5.0 × 55 cm) (flow rate, 15 mL/h; fraction, 15 mL/tube) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) beforehand. After rinsing thoroughly with the equilibrating buffer, linear gradient elution was conducted with 1500 mL of 20 mM Tris-HCl (pH 7.5) in the mixing chamber and 1500 mL of 0.4M NaCl in 20 mM Tris-HCl (pH 7.5) in the reservoir chamber. As shown in Figure 1, BGLs were divided into two components (BGL1, 61 mL; BGL2, 142 mL). The purification step hereinafter was conducted separately for the BGL1 and BGL2 fractions (designed as *step* 5-2).

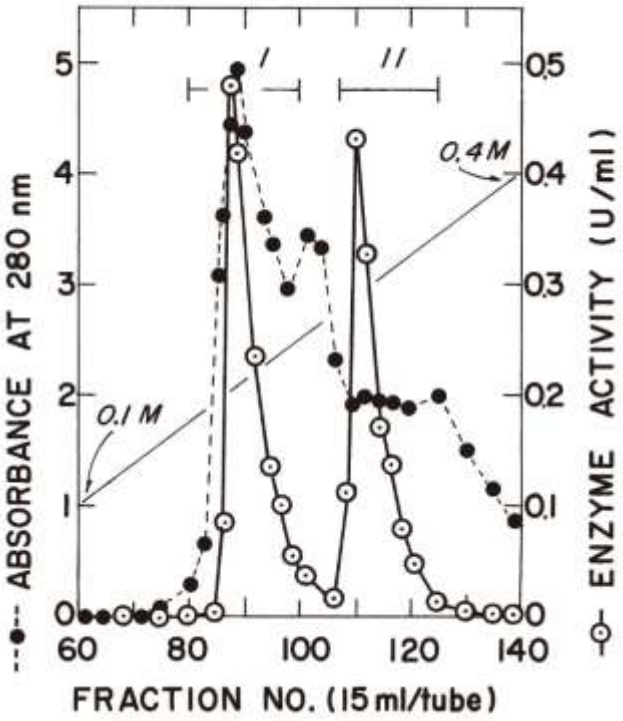

Fig. 1. Elution profiles of *T. curvata* KP1246 β-glucosidase and of protein from a DEAE-Sepharose column (Step 5). The dialyzed fraction of Sephadex G-75 eluate (Step4) was applied to DE-AE-Sepharose column and eluted as described in Materials and Methods. The linear 0.1-0.4 M NaCl gradient elution was started at the site of fraction 60. The activity (○) is expressed in U/ml eluate. The A_{280} of each fraction (●) was measured as an indication of protein content. Ⅰ, BGL1; Ⅱ, BGL2.

BGL1 fraction.

Step6-1 Phenyl-Sepharose hydrophobic chromatography. Solid ammonium sulfate was added to 61 mL of the BGL1 solution obtained in Step 5 to obtain ammonium sulfate precipitate with a final concentration of 75% saturation. This was dialyzed for 24 h in 5 mM PPB (pH 6.8)/0.8M $(NH_4)_2SO_4$/0.02% sodium azide. After centrifugation, the clear supernatant obtained was applied to the phenyl-Sepharose column (2 × 30 cm) equilibrated with the aforementioned buffer. The fraction was obtained at the flow rate of 15 mL/h and 5 mL/tube. After rinsing thoroughly with the same buffer, linear gradient elution was conducted with 500 mL of 20 mM PPB/0.8M $(NH_4)_2SO_4$ in the mixing chamber and 500 mL of 20 mM PPB/50% ethylene glycol in the reservoir chamber. Activity fractions (tube numbers 65–85) were retrieved (volume: 70 mL).

Step7-1 Bio-gel P-150 gel column chromatography. For the BGL1 fraction obtained in Step 6-1, buffer exchange and concentration operation were repeated 3 times on Amicon PM-10 membrane using 10 mM PPB (pH 6.8)/0.02% sodium azide. This solution was dialyzed in 10 mM PPB (pH 6.8)/0.02% sodium azide. After centrifugation, 2.0 mL of concentrated BGL1 obtained was applied to the Bio-gel P-150 column (1.9 × 99.5 cm) equilibrated with the aforementioned buffer. The fraction was obtained at the flow rate of 14 mL/h and 2.0 mL/tube. Activity fractions (tube numbers 67–90) were obtained (69 mL).

Step8-1 Bio-gel P-150 gel column chromatography. For the BGL1 activity fraction obtained in Step 7-1, concentration was performed in the same manner as Step 7, and 2.0 mL of enzyme fraction obtained after centrifugation was applied to the Bio-gel P-150 column (1.9 × 99.5 cm) equilibrated with thebeforehand mentioned buffer. The fraction was

obtained at the flow rate of 14 mL/h and 2.0 mL/tube. Activity fractions (tube numbers 68–85) were obtained (54 mL). These fractions were used as the final BGL1 specimen.

BGL2 fraction

Step6-2 Phenyl-Sepharose hydrophobic chromatography. From the BGL2 solution retrieved in Step 5-2 (142 mL), the ammonium sulfate precipitate with a final concentration of 75% saturation was obtained. After performing the same procedures as in Step 6 of BGL1, this ammonium sulfate precipitate was applied to phenyl-Sepharose column (2 × 30 cm), and the fraction was obtained at the flow rate of 15 mL/h and 5 mL/tube. Linear gradient elution was conducted with 500 mL of 20 mM PPB/0.8M $(NH_4)_2SO_4$ in the mixing chamber and 500 mL of 20 mM PPB/50% ethylene glycol in the reservoir chamber. Activity fractions (tube numbers 60–97) were retrieved (volume, 111 mL).

Step7-2 Bio-gel P-150 gel column chromatography. After concentrating with Amicon PM-10 membrane, the BGL2 fraction obtained in Step 6-2 2.2 mL of the resulting solution was gel filtrated with the Bio-gel P-150 column (1.9 × 99.5 cm). Activity fractions (tube numbers 66–91) were obtained (74 mL).

Step 8-2 DEAE-Sepharose ion-exchange chromatography The activity fraction obtained in Step 7-2 was dialyzed in 20 mM Tris-HCl buffer (pH 7.5) and applied to DEAE-Sepharose column (2.0 × 25 cm) (flow rate: 15 mL/h, fraction: 2 mL/tube) equilibrated with a 20 mM Tris-HCl buffer (pH 7.5) beforehand. After rinsing thoroughly with the equilibrating buffer, linear gradient elution was conducted with 0–0.2M NaCl. Conditions were as follows: mixing chamber, 150 mL of 20 mM Tris-HCl (pH 7.5); reservoir chamber, 150 mL 0.2M NaCl in 20 mM Tris-HCl (pH 7.5). Eighty-four milliliters of BGL2 activity fraction was obtained.

Table 1. Purification of *T. curvata* KP1246 BGL1

Purification steps	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purity
1. Culture broth	25100	28523	305	0.0107	100	1.0
2. Ammonium sulfate fraction	163	7305	143	0.0196	46.8	1.83
3. Acetone fraction	87.8	2677	89.1	0.0332	29.2	3.10
4. Sephadex G-75 eluate	330	1339	88.2	0.0658	28.9	6.15
5-1. DEAE-Sepharose eluate	61	416	75.8	0.180	24.8	16.8
6-1. Phenyl-Sepharose eluate	70	5.4	33.0	6.11	10.8	571
7-1. Bio-Gel P-150 eluate	69	2.2	24.1	11.0	7.90	1028
8-1. Bio-Gel P-150 eluate	54	1.0	23.4	23.4	7.67	2186

Step9-2 Bio-gel P-150 gel column chromatography. For the BGL2 activity fraction obtained in Step 8-2, concentration and centrifugation was performed with Amicon PM-10 membrane. The obtained solution (1.9 mL) was gel filtrated with the Bio-gel P-150 column (1.9 × 99.5 cm). Fifty-nine milliliters of activity fraction was obtained, and this fraction was used as the final BGL2 specimen.

Electrophoresis and electrofocussing. The sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run according to

Table 2. Purification of *T. curvata* KP1246 BGL2

Purification steps	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purity
1. Culture broth	25100	28523	305	0.0107	100	1.0
2. Ammonium sulfate fraction	163	7305	143	0.0196	46.8	1.83
3. Acetone fraction	87.8	2677	89.1	0.0332	29.2	3.10
4. Sephadex G-75 eluate	330	1339	88.2	0.0658	28.9	6.15
5-2. DEAE-Sepharose eluate	142	162	36.0	0.172	11.8	16.8
6-2. Phenyl-Sepharose eluate	110	68.2	42.2	0.618	13.8	57.8
7-2. Bio-Gel P-150 eluate	74	16.9	32.0	1.89	10.5	177
8-2. DEAE-Sepharose eluate	84	2.45	7.80	3.20	2.56	299
9-2. Bio-Gel P-150 eluate	59	0.89	9.7	10.9	3.20	1019

the methods of Laemmli (27). The SDS gel contained 10% acrylamide. Electrophoresis was carried out for 3h at 25mA/gel and at 25℃. After electrophoresis, the gels were stained with Coomassi brilliant blue for 90 min and decolorized with 25% ethanol/8% acetic acid.

Twenty µl each of BGL1 or BGL2 was applied to native agarose gel with a pH range of 3.5 - 10.0. The carrier ampholytes employed is Ampholine pH3.5-10 (LKB-ProdukterAB, Sweden). After electrophoresis at 4℃ for 90 min, according to the method of Weber and Osborn (28), the gels were sliced at 2 mm intervals. Each of the gel section was homogenized in 500 µl of pure water then left standing for 10 minutes. The enzyme activity was measured for 100 µl of the supernatant, and the pH of the remaining supernatant was obtained (29).

Analytical gel filtration BGLs were subjected to gel filtration on a Bio-Gel P-200 column (1.5 x 99.5 cm) with PBB/0.5 M NaCl/0.02% sodium azide, previously calibrated with the proteins of known *M*r and Stokes radius. The standards used were: lactate dehydrogenase from rabbit muscle, bovine serum albumin, peroxidase from horse radish,

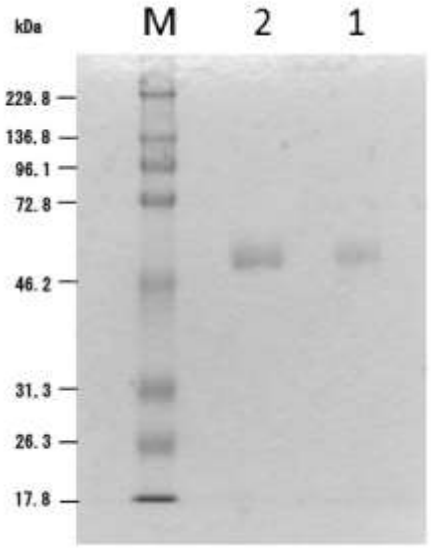

Fig. 2. Electrophoresis of purified KP1246 BGL1 and BGL2. Electrophoresis was performed at 25℃ for 3 h at 25 mA/gel, using 3 µg BGL1 and 5µg BGL2 proteins, respectively. Lane M denotes *M*r weight standards; lane 1, BGL1; lane2, BGL2. The *M*r weight standards used (from the top to the bottom in the figure) were myosin (229800), β-galactosidase (136800), phosphrylase-b (96100), bovine serum albumin (72800), ovalbumin (46200), carbonic anhydrase (31300), trypsin inhibitor (26,300) and lysozyme(17800).

chicken egg albumin, beef pancreatic α–chymotrypsinogen, and human hemoglobin. The *M*r of BGLs was estimated by the method of Andrew (30), and the Stokes radius by the method of Bumett (31).

Each $s_{20,w}$ of BGLs was estimated by the methods of Martin and Ames (32). The enzymes, along with the $s_{20,w}$ markers, were centrifuged at 7℃ for 21 h at 36000 rpm in a Hitachi 65P ultracentrifuge and a swing rotor RPS-50. The markers applied were: lactate dehydrogenase from rabbit muscle, peroxidase from horse radish and cytochrome *c* from horse radish and *B. amyloliquefaciens* KP1071 exoα-1,4-glucosidase (24).

Activity and stability dependent on pH. BGL activity was assayed as in Materials and methods, except that cellobiose was used as the substrate and that pH of the reaction mixture was changed by using 0.05 ml of the following buffers: 0.1M acetate (pH1.0-4.1), 0.1M succinate (pH3.8-5.5), 0.1M maleate (pH5.5-6.8), 0.1M PPB (pH 6.0-8.5), and 0.1M glycine–NaOH (pH7.5-12.0).

Either BGL1 or BGL2 (2.0 U/ml) was mixed with the equal volume of one of the buffers as described above. After incubation for 16 h at 60℃, each of the mixtures was 10-fold diluted with 50 mM PPB (pH 6.8), respectively. The mixtures were determined for glucosidase activity, as described above.

Effects of metal ions, EDTA and SH-reagents. The final preparations of BGL1 and BGL2 were exhaustively dialyzed against 10mM PPB and diluted up to 100-fold with PPB. Respective enzyme activities were assayed as described above, except that the reaction mixture contained one of metal ions (as chlorides) and EDTA. SH-reagents (each 45μM in the final reaction medium) were pre-incubated with BGL1 or BGL2 for 30 min at 55℃ in 10 mM PPB before the enzyme assay.

Results and discussion

For BGL1, culture supernatant was purified through 8 steps by the factor of 2,186 at the recovery rate of 7.7%, and as a result, 1.0 mg of specimen with specific activity of 23.4 U/mg protein was obtained (Table 1). For BGL2, the culture supernatant was purified through 9 steps by the factor of 1,019 with the recovery rate of 3.2%, and as a result, 0.89 mg of specimen with specific activity of 10.9 U/mg protein was obtained (Table 2). A variety of physicochemical methods were employed to determine the size and charge of these BGLs. The *M*r of BGL1 and BGL2 was estimated to be 51,000 Da and 50,000Da, respectively by SDS-PAGE (Fig. 2). No carbohydrates were detected in the each enzyme preparations by the phenol/sulfuric acid method (33). The relationship between weight-average *M*r and $S_{20,w}$ of the purified enzymes was indicative of

Table 3. Molecular properties of *T. curvata* KP1246 BGL1 and BGL2[a)]

	BGL1	BGL2
*M*r (SDS-PAGE)	51000	50000
(Gel filtration)	50000	44000
$S_{20,w}$	4.4 S	4.2 S
p*I*	5.4	4.2
Stokes radius (nm)	2.92	3.06

a) BGL1 and BGL2 along with S20,w markers, were centrifuged, as shown in Materials and methods. The makers used were pig heart lactate dehydrogenase (7.0S), *B. coagulans* α-glucosidase (4.8S), *Bacillus thermoamyloliquefaciens* KP1071 α-glucosidase I (4.0S), and horse heart cytochrome C (2.0S). The molecular mass and Stokes radius markers used to calibrate the gel filtration column were γ-globulin (5.54 nm), pig heart lactate dehydrogenase (4.29 nm), bovine serum albumin (3.64 nm), egg albumin (2.37 nm) and human hemoglobin (3.13 nm) and α-chymotrypsinogen (2.26 nm).

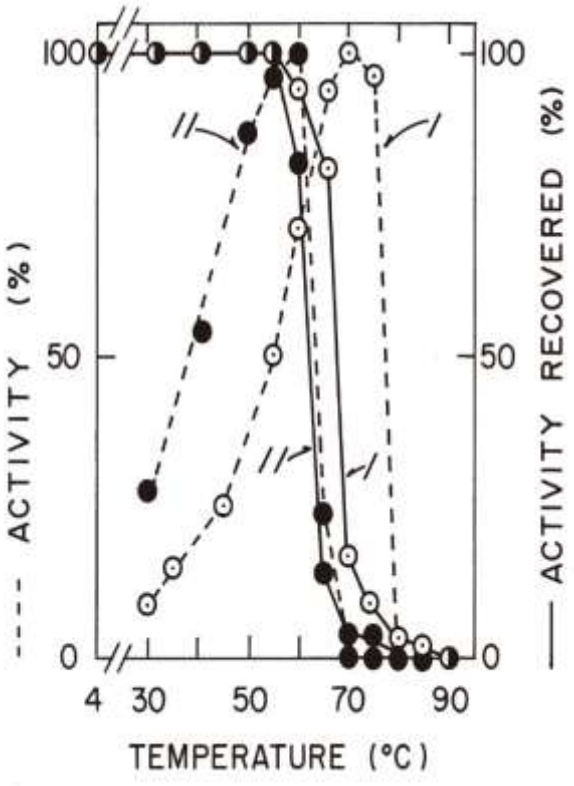

Fig. 3. Effects of temperature on the activity and stability of KP1246 BGL1 and BGL2.
The activities of BGL1 and BGL2 were determined as described in Materials and methods, except that the temperature was changed (----). The maximal activity is taken as 100%. BGL1 (0.12U/ml) or BGL2 (0.18 U/ml) was incubated for 30 min at different temperatures (———) in 50 mM PPB (pH6.8). The mixture was assayed for remaining activity. The activity observed after the incubation at 4℃ is indicated as 100%. I and ○, BGL1; II and ●, BGL2.

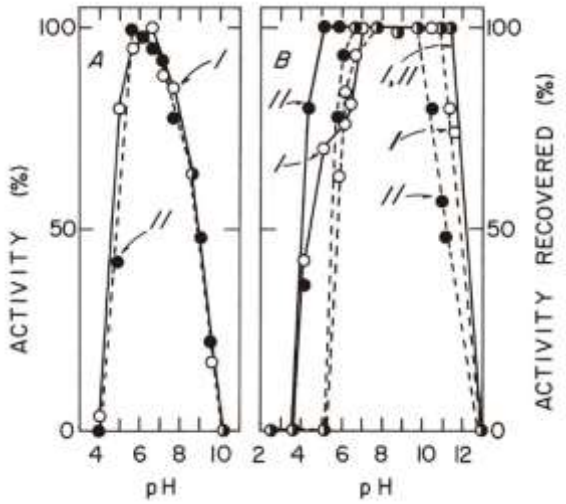

Fig. 4. Effects of pH on the activity (A) and stability (B) of KP1246 BGL1 and BGL2.
In (A), the activities of BGL1 (○) and BGL2 (●) at different pHs were determined as described in "Activity and stability dependent on pH" in Materials and methods. The maximal activity is taken as 100%. In (B), BGL1 or BGL2 was treated for18 h at 25℃ (———), or at 60℃, as described in "Activity and stability dependent on pH" in Materials and methods. The mixture was assayed for remaining activity. The activity found after the incubation for 18 h at pH 6.8 and at 4℃ is expressed as 100%. I and ○, BGL1; II and ●, BGL2.

Table 4. Effects of metal ions, EDTA and SH-reagents on the activity of *T. curvata* KP1246 BGL1 and BGL2

Addition	Final concentration (mM)	Inhibition % of activity[a)] BGL1	BGLI2
None		0	0
Hg^{2+}	2.0	100	100
Cu^{2+}	2.0	43.0	74.7
Ni^{2+}	2.0	44.2	16.7
Cd^{2+}	2.0	35.5	28.6
Co^{2+}	2.0	24.4	24.4
Sn^{2+}	2.0	17.4	33.9
Zn^{2+}	2.0	16.9	32.8
Ca^{2+}	2.0	8.1	34.2
Sr^{2+}	2.0	1.2	44.5
Pb^{2+}	2.0	0	25.2
Fe^{2+}	2.0	0	22.3
Ba^{2+}	2.0	0	20.9
Mn^{2+}	2.0	0	17.5
Mg^{2+}	2.0	0	10.5
EDTA	2.0	0	26.1
p-CMB	0.045	100	32.5
DTNB	0.045	12.8	33.9

p-CMB, *p*-chloromercuribennzoate; DTNB, 5,5'-dithio-bis(2-nitrobenzoate)

[a)]The activity was determined as in Materials and methods, except that the reaction mixture (1.0 ml) contained 30 mM PPB, 2 mM metal ions or EDTA and 20 mU of BGL1 or BGL2 (24). SH-reagents was incubated with respective enzymes at 55℃ for 30 min before the addition of the substrate.

homogeneity and absence of protein aggregates (Table 3). The *M*r of the BGLs agreed fairly well with those obtained by Stokes radius, respectively (Table 3). Together these results indicated that BGL1 and BGL2, respectively was homogeneous and was a single, monomeric polypeptide (Table 3, Fig 2). The considerable difference in the *p*I values of these 2 enzymes clearly indicated that BGL1 and BGL2 could only be distinguished after separation (step 5) using DEAE-Sepharose chromatography because of the similarity in their molecular properties (Fig. 1, Tables 1 and 2).

BGL1 and BGL2 were most active at 70℃, and at 55℃, respectively (Fig. 3). BGL1 exhibited more stability to heat resistance between 55°C and 80℃. Optimum

Table 5. Kinetic characteristics of BGL1 and BGL2 from *T. curvata* KP1246

	BGL1		BGL2	
	Ki **(mM)**	**Type of inhibition**	***Ki*** **(mM)**	**Type of inhibition**
Tris	**7.5**	**C***	**44**	**NC**
Glucose	**No inhibition**		**408**	**NC**
Cellobiose	**50**	**NC****	**15.6**	**NC**

*: competitive, **: non-competitive

The *p*NPG hydrolyzing activity was assayed as described in Materials and methods, except that one of the compounds listed in the table was added in the reaction mixture. The activity was not inhibited by the following compounds; glycine, ammonium chloride, aniline (each 5 mM). *Ki* values were determined by the method of Dixon (39).

temperature for the reaction of BGL1 was 15°C higher than that of BGL2, and a wider pH range was obtained for BGL1 in regard to heat resistant temperature (Fig.4). The temperature optimum of activity of BGL1 was at 70°C, which was 8-10°C higher than that of BGL2 (Fig. 3). BGL2 lost all of the activity at 70°C in 30 min, whereas was slightly higher than that of BGL1, showing that BGL2 tolerates some denaturants more strongly than BGL1. Moreover, it was found that the advantage of BGL2 is in the lower susceptibility to inhibition by heavy metal ions than that of BGL1 (Table4).

BGL1 kept its activity 35% of the original one. On the other hand, BGL2 was more resistant to low pH range than BGL1 after incubating for 18h (Fig. 4). Moreover, BGL2 tolerates urea, ethanol and SDS more strongly than BGL1(Fig. 5). Half-life of BGL2 for 5h at 55℃ was depicted 0.05% SDS, 21% ethanol or 3.8 M urea, respectively. Each value

The results together showed that BGL2 was most thermostable and tolerant of denaturants than the other β-glucosidases reported among thermoactinomyces (6, 34). Production of cellooligosaccharides from cellulose was slower for thermophilic actinomycete

KP1246 in comparison to mesophilic filamentous bacteria (35, 36). However, as a result of this study, the *Ki* value of glucose to BGL2 was found to be high (408 mM), suggesting that inhibition by glucose occurs slowly (Table 5). In addition, it was found that inhibition by glucose does not occur at all for BGL1 (Table 5). These results suggest that thermophilic actinomycete is superior to mesophilic bacteria from *Phanerochaete chrysosporium* (35), *Trichoderma reesei* (36), and *Fomitopsis palustris* (37, 38) in that the level of production inhibition by glucose is not as high (21, 22).

Mesophilic filamentous bactria BGL degrades cellooligosaccharides or cellobiose into glucose as the final stage of cellulase complex and most of BGL were produced outside the bacterial cell. To investigate celobiose degradation in the thermophile, kinetic parameters were determined using *p*NPG, cellobiose and other compounds (Table 5 and 7). The K_m and V_{max} or K_i values were determined by the method of Dixon (39) and Lineweaver and

Table 6. Substrate specificity of *T. curvata* KP1246 BGL1 and BGL2

Substrate	Linkage	Final concentration	Glucose formation (μmol/min/mg protein)	
			BGL1	BGL2
Gentiobiose	β-1,6	10 mM	2.0×10^{-3}	2.4×10^{-4}
Lactose	β-1,4	10 mM	5.7×10^{-4}	2.2×10^{-6}
Arubutin	β-1,4	10 mM	ND	ND
Avicel	β-1,4	0.10%	ND	ND
CM-cellulose	β-1,4	0.10%	ND	ND

ND: not hydrolyzed
Hydrolysis activities were determined from the amount of released glucose as described in Table 4, except that the final concentration of the substrates was 10 mM or 0.10% as shown in the table. Glucose formation was not detected when the following sugars were used as substrates: maltose, isomaltose, amylose A, amylopectine, starch, sucrose and dextrin (10 mM each).

Burk (40), respectively. The *Km* value of BGL to cellobiose is around a few millimoles (Table 7); however, the inhibition constant (*Ki* value) of cellobiose to cellulase is not more than 100 μM (34). Among the substrates tested, the highest catalytic efficiency (*Vmax*/*Km*) and lowest *Km* values were obtained for *p*NPG, suggesting that BGL1 is a typical β-glucosidase (Tables 5 and 7). Tris reagent inhibited BGL1 in a typical competitive manner, as demonstrated in *Bacillus* α-glucosidase (24). On the other hand, Tris inhibited BGL2 in a non-competitive manner (Table 5). Compared with *p*NPG, cellobiose was not only a poor substrate for both BGL1 and BGL2, but also acted as an inhibitor of glucosyl-β-glucoside substrates (Table 7). Glucose did not inhibit the glucosyl-β-glucoside hydrolysis activity of BGL1, and it acted as a poor inhibitor BGL2 (Table 6). Other substrate bearing β-1,4-linkage, such as lactose, also acted as poor substrates (Table 6). Other sugars with higher degree of polymerization (DP) of the glucose unit, such as Avicel or CM-cellulose were not hydrolyzed by both BGL1 and BGL2 (Table 6). This is typically observed for β-glucosidase,

Table 7. Substrate specificity of *T. curvata* KP1246 BGL1 and BGL2

Substrate	BGL1			BGL2		
	Km **(mM)**	***Vmax*** **(μmol/min/mg protein)**	***Vmax/Km***	***Km*** **(mM)**	***Vmax*** **(μmol/min/mg protein)**	***Vmax/Km***
***p*NPG**	1.25	25.0	20.0	2.30	10.0	4.3
Cellobiose	2.70	3.9×10^{-2}	1.4×10^{-2}	1.90	1.2×10^{-2}	5.2×10^{-3}
Salicin	19.4	1.5×10^{-2}	7.7×10^{-4}	2.0	3.3×10^{-3}	1.7×10^{-3}
Amygdalin	6.3	9.0×10^{-3}	1.4×10^{-3}	not hydrolyzed		

Salicin, 2-(hydroxymethyl)phenyl -β- D-glucopyranoside: Amygdalin, (R) -α-[(6-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]- benzeneacetonitrile.
BGL1 and BGL2 (38 mU/1.0 ml reaction mixture) were incubated for 30 min with one of various substrates and hydrolytic rates were estimated from the amount of glucose released from the substrates was as described in Materials and methods. The *Km* and *Vmax* of BGL1 and BGL2 were determined by the method of Lineweaver and Burk plot, respectively (40). The values are means of two independent experiments performed in duplicates.

which shows an affinity for oligosaccharide substrates with a DP of about 2 or 3. Similarly, gentiobiose which contains β-1,6-linkage and β-1,4-linkage, respectively, was poor substrate for both enzymes (Table 6). BGL1 and BGL2 exhibited a different behavior towards amygdalin, which comprises β-1,6-linkage like that in gentiobiose; amygdalin likely acted as a poor substrate because of the benzeneacetonitrile group added to its structure, which could lead to poor recognition of amygdalin as a substrate.

Thermophilic actinomycete cellulase complexes reported to date were classified into EG, CBH, and BGL, according to the cellulose degradation format based on the substrate specificity and products. However, as the information on the base sequence of the cellulase gene is accumulated, classification of cellulase by hydrophobic cluster analysis was analyzed (41, 42), and elucidation of the 3D structure of cellulase resulted in the classification based on protein structure (clan) being established as the higher classification of the cellulase family (43, 44). Though this is established for BGL derived from other sources, the method of classification where the family name is included in the gene name is not yet applied for thermophilic actinomycetes.

The order *Thermomonospora* has been integrated into 7 groups, which include *Thermomonospora curvata* and *Thermobifida fusca* (45). Furthermore, the whole genetic structure of the type strain B9^{T} of *T. curvata* was presented (46). Initially, we reported that the separately obtained pectinolytic actinomycete KP1280 belonged to *T. fusca* (47); however, as a result of partial 16SrDNA analysis, this was corrected to *Thermobifida fusca*, and as a result of further complete 16SrDNA analysis, it was found to belong to *Thermobifida* sp., which is most closely related to *Thermobifida fusca* (48). In order to clarify whether KP1246 belongs to the or-

der *Thermobifida* or *Thermomonospora*, it is necessary to wait for a detailed cluster analysis to be conducted. However, there is a need for similar integration to be performed for *T. curvata* KP1246 in the near future.

As a result of this study, it was discovered that *T. curvata* KP1246 is able to express a complete cellulase complex where two types of BGL, which have completely different heat-resistance properties and inhibition format, act to complement each other, with only a single bacterium. A similar complete single cellulase complex is proposed for thermophilic anaerobe, *Clostridium phytofermentans* (49), and for thermophilic actinomycete (50); however, it is expected that the combination of the closely related species KP1246 (cellulase system) and KP1280 (pectinase system) in an efficient manner, under aerobic and high-temperature conditions to

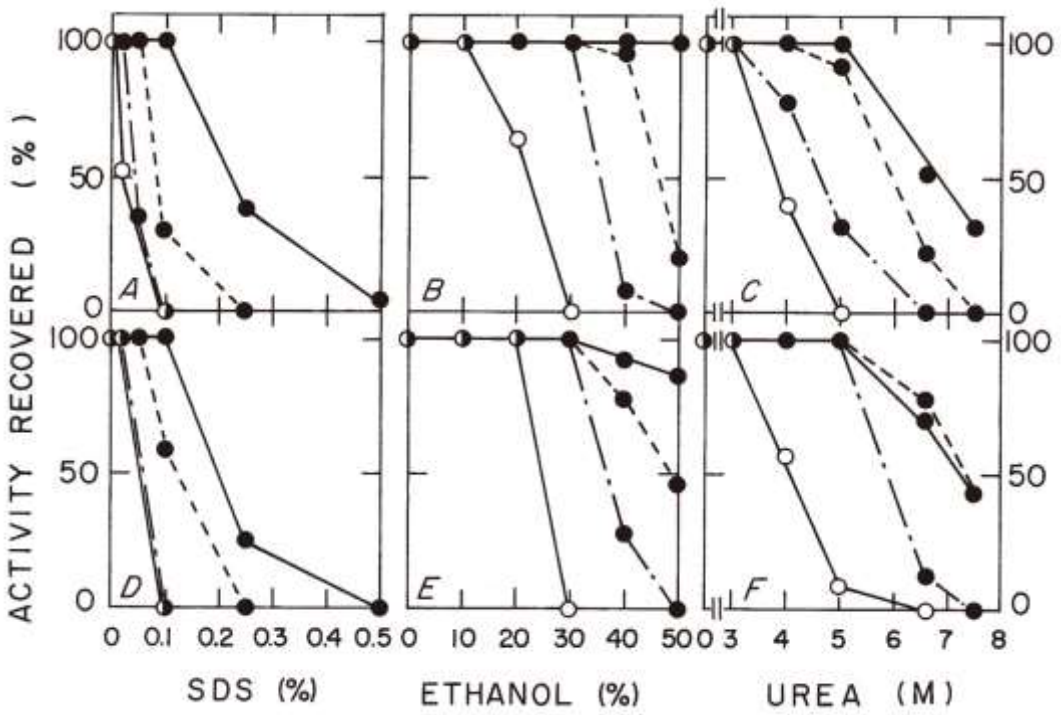

Fig. 5. Effects of SDS, ethanol and urea on the stability of KP1246 BGL1 and BGL2. BGL1 (1.2 U/ml) and BGL2 (2.1 U/ml) were incubated for 5 h at 4℃ (—●—), or at 25℃ (--●--), or at 37℃ (-● -●- ●-) or at 60℃ (—○—) in 50 mM maleate buffer (pH 6.8). The activities of the untreated enzymes are taken as 100%. A,B,C, BGL1; D,E,F, BGL2.

enable easier handling, will enable the establishment of a new bioenergy production system from unused plant biomass.

These results seem to show that BGL1 and BGL2 were not β-glucosidases, but were bifunctional enzymes exhibiting exo-β-(1,3)/(1,6) and endo-β-(1,4) activity towards β-glucans, which could not be assigned to any existing Enzyme Commission groups (51). The amino acid sequence showed high identity to hypothetical proteins within the fungal taxa and thus were defined as GH131, a new family of glycoside hydrolases (44, 45). Another candidate for BGL1 and BGL2 was *Bacillus* lichenase (1,3-1,4-β-Glucanases: EC 3.2.1.73), which hydrolyzed linear-β-glucans containing β-1,3 and β-1,4 linkages, such as cereal-β-glucans and lichenan, with a strict cleavage specificity for-β-1,4 glycosidic bonds on 3-*O*-substituted glucosyl residues (51, 52). The structure–function aspects of the enzymatic action of bacterial 1,3-1,4-βglucanase (53) or *Streptomyces* 1,3-β-glucanase (54) could be applied to BGL1 and BGL2. Hydrophobic interactions are considered predominantly responsible for protein thermostability, whereas hydrogen-bonding and chargecharge interactions weakened at elevated temperatures (55). Based on the previous data for the phenotypic characteristics of the strain (21, 22), KP1246 can be classified as *T. curvata*.

Addendum

While this manuscript on β-glucosidase derived from KP1246 strain was being peer-reviewed by referees, we were informed that the KP1246 strain deposited at the Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto 606-8522, Japan had been sterilized and discarded along with KP1244 and KP1245 strains, which had been developed around the same time as the KP1246 strain. This happened following our making a request as a developer to the Kyoto

Prefectural University for the transfer of these 3 strains.

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