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Molecular Cloning and Characterization of the Gene Encoding Cold-Active β-Galactosidase from a Psychrotrophic and Halotolerant *Planococcus* sp. L4

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The gene *bgaP* encoding cold-active β-galactosidase from a psychrotrophic and halotolerant *Planococcus* sp. L4 was cloned into *Escherichia coli* and sequenced. A sequence analysis of the DNA responsible for the BgaP gene revealed an open reading frame of 2031 bp encoding for a protein of 677 amino acid residues. The BgaP was heterologously expressed in *E. coli* and purified followed by Ni^{2+} affinity chromatography. The molecular mass of the native enzyme was approximately 156 kDa as determined by gel filtration. The results of sodium dodecyl sulfate−polyacrylamide gel electrophoresis and the deduced amino acid sequence of the BgaP indicated molecular masses of 78 and 77.311 kDa, respectively, suggesting that the BgaP is a dimer. The purified BgaP had an isoelectric point of 4.8 and exhibited maximal activity at 20 °C and pH 6.8 under the assay conditions used. The enzyme is particularly thermolabile, losing all activity in only 10 min at 45 °C. It was able to hydrolyze lactose as a substrate, as well as *o*-nitrophenyl-β-D-galactopyranoside (ONPG); the K_m values with ONPG and lactose were calculated to be 5.4 and 20.4 mM at 5 °C, respectively. The catalytic efficiencies of BagP for lactose at 5 and 20 °C had 14 and 47 times more than that of *E. coli* β-galactosidase at 20 °C, respectively. Therefore, cold-active β-galactosidase from the psychrotrophic and halotolerant *Planococcus* sp. L4 could conceivably be developed to fulfill the practical requirements to enable its use for lactose removal in milk and dairy products at low temperature or a reporter enzyme for psychrophilic genetic systems.

KEYWORDS: Cold-active β-galactosidase; gene cloning; properties; psychrotrophic and halotolerant *Planococcus* sp. L4

INTRODUCTION

Cold-active enzymes are produced by psychrophilic and psychrotrophic organisms living in low-temperature environments and defined by high catalytic activity at low temperature with lower activation energy than those of mesophilic counterparts. To obtain high catalytic efficiency, cold-active enzymes have probably evolved to have high conformational flexibilities, although stability has been sacrificed. It is thought that the flexible structures of these enzymes mainly consist of a decrease in the number and strength of intramolecular interactions and in some cases in a better accessibility of the catalytic cavity. As compared to mesophilic enzymes, these enzymes display three general distinguishing characteristics: a higher specific activity (k_{cat}) or a catalytic efficiency (k_{cat}/K_m) at temperatures between 0 and 30 °C, a low optimal temperature for activity, and limited stability in the presence of thermal increases and denaturing agents (*1*). Therefore, interest in cold-active enzymes has been increasing in recent years, and various candidates have been identified for a huge biotechnological potential in food processing, biomass conversion, molecular biology, environmental biosensors, bioremediation, and cleaning of contact lenses (*2*, *3*); in particular, they are attractive in food industries such as the processing of fruit juices and milk. There is an industrial trend to treat food stuffs under mild conditions to avoid spoilage and changes in taste and nutritional values at ambient temperature (*4*, *5*).

β-Galactosidases (EC 3.2.1.23) catalyze the hydrolysis of β-1,4-D-galactosidic linkages. This enzyme is distributed in numerous microorganisms, plants, and animal tissues. In view of the applications of β-galactosidase in both dairy industry and biotechnology, these glycosyl hydrolases have become important enzymes (*6*). The application of cold-active β-galactosidase to the hydrolysis of lactose in dairy products, such as milk and

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cheese whey, has received much attention (*7*). It can be used to degrade lactose for several purposes: (i) removal of lactose from refrigerated milk for people who are lactose intolerant; (ii) conversion of lactose to glucose and galactose, which are more fermentable sugars than lactose, in whey; and (iii) removal of lactose from pollutions of the dairy industry. Therefore, a great deal of efforts has been made to screen and isolate novel β-galactosidases from different sources in recent years including *Pseudoalteromonas* sp. 22b (*8*), *Pseudoalteromonas haloplanktis* (*9*), *Bacillus subtilis* KL88 (*10*), *Carnobacterium piscicola* BA (*11*), *Arthrobacter* sp. C2-2 (*12*), *Arthrobacter psychrolactophilus* (*13*−*15*), *Arthrobacter* isolate SB (*16*), and *Planococcus* sp. (*17*). However, most of the cold-active β-galactosidases previously reported showed optimal activity about 25−40 °C under alkaline conditions and practically low activity at 0 °C. An ideal cold-active β-galactosidase for treating milk would work well at 4−8 °C; be active at pH 6.7−6.8; not be inhibited by sodium, calcium, or galactose; and be specific for lactose. Currently, applied to lactose hydrolysis, mesophilic *Kluyveromyces lactis* β-galactosidase has a temperature optimum of 35 °C and performs poorly at 20 °C, requires manganese or magnesium and potassium or sodium, and is inhibited by calcium at concentrations greater than 0.1 mM and by galactose with a K_i of 42 mM (*13*); its replacement with a higher level of counterpart shortens the process of lactose cleavage and runs cold to eliminate any contamination with mesophilic microflora, to avoid nonenzymatic browning products, formed at higher temperature. In the present study, we carried out the enzymatic and molecular characterization of a novel cold-active β-galactosidase from psychrotrophic and halotolerant *Planococcus* sp. L4 to resolve these problems described above. Aside from their importance in treating milk products during shipping and storage, cold-active β-galactosidases are also useful for investigations of structural basis of protein stability by comparison and offer great potential for biotechnological application because they have been studied extensively and have been characterized in a number of organisms. We compared the activity and structural characteristics of the newly purified enzyme to those of homologous enzymes from mesophilic organisms in an attempt to clarify features unique to cold activity.

MATERIALS AND METHODS

Chemicals and Reagents. All chromogenic nitrophenyl analogues were purchased from Sigma. All other chemicals and reagents were of analytical grade and were purchased from commercial sources, unless otherwise stated.

Isolation and Culture Conditions. The psychrotrophic and halotolerant *Planococcus* sp. L4 was isolated from surficial saline soils near Daqing oil field in China (46° 58′ N, 125° 03′ E). The strain was purified by the dilution-to-extinction approach by using modified Luria−Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 50 g of NaCl in 1 L at pH 7.0). *Planococcus* sp. L4 was grown on modified LB medium with 2% lactose and 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to test for the presence of β-galactosidase activity. Physiological and biochemical characteristics were performed according to the manual of identification for general bacteriology (*18*). Cell morphology and dimensions were observed using phase contrast microscopy. Colony morphology was determined using a Leica M8 stereomicroscope. Gram staining was carried out according to Dussault (*19*). The KOH test was confirmed according to Halebian et al. (*20*).

16S rRNA Gene Amplification and Cloning of β-Galactosidase Gene. Routine DNA manipulation was carried out as described by Sambrook et al. (*21*). Restriction enzymes and DNA polymerase were purchased from Takara (Dalian, China). Each enzyme was used according to the recommendations of the manufacturer. DNA ligations were performed using T4 DNA ligase (Fermentas). Genomic DNA and plasmid DNA were isolated using genomic and plasmid DNA isolation kits, respectively (QIAamp DNA Mini Kit, E.Z.N.A. Plasmid Miniprep kit I). A DNA purification kit (E.Z.N.A. Gel Extraction kit) was used to recover DNA fragments from agarose gels. Molecular identification was done by polymerase chain reaction (PCR) and DNA sequencing. The 16S rRNA gene sequence was determined by direct sequencing of the PCR product. Genomic DNA extraction, amplification of the 16S rRNA gene, and purification of the PCR products were carried out as described previously (*22*). Approximately 1.5 kb of PCR product was directly sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. Sequence reactions were electrophoresed using the Applied Bio systems 373A DNA Sequencer. To prepare genomic DNA fragments of approximately 2−6 kb, genomic DNA from *Planococcus* sp. L4 was subjected to partial digestion with Sau3AI and fractions containing DNA fragments of the desired size were pooled, and the resultant DNA fragments were ligated into pUC19Δ lacZ (kindly provided by Petra Karasova-Lipovova), which had been previously digested with *Hind*III and dephosphorylated. *Escherichia coli* JM109 was transformed with the library and plated onto LB agar plates containing 100 μg/mL of ampicillin, 0.01% X-Gal, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). β-Galactosidase-positive colonies appeared blue at 20 °C after 2 days. Plasmid DNA from one of these transformants was purified, and the insert was analyzed on a ABI 377 DNA sequencer. The complete double-stranded sequence was obtained and used in the comparison. Nucleotide and deduced amino acid sequence analyses, open reading frame search, multiple alignment, molecular mass, and isoelectric point calculations were performed using DNASIS software. A database homology search was performed with BLAST program provided by NCBI.

Expression and Purification of Recombination β-Galactosidase. The novel β-galactosidase gene (*galP*) was amplified by a PCR with the primers galP-f (5′-CACGGATCCATGATCAACGATAAATTGCCGAAGATTTG) and galP-r (AGCAAGCTTATACTTTCGCTAAGATGAAGACGC), containing restriction enzyme sites (underlined) for *BamH*I and *Hind*III, with the shortest DNA fragment isolated from β-galactosidase-positive transformant as a template. The PCR product was digested with *BamH*I and *Hind*III, ligated into the *BamH*I- and *Hind*III-linearized expression vector pET-22b(+), and introduced into *E. coli* BL21 (DE3). Transformants were cultivated at 18 °C in LB medium containing 100 μg/mL ampicillin to an optical density at 600 nm of 0.5−0.6, expression of the enzyme was induced by IPTG to a final concentration of 1 mM, and the cells were further cultivated at 18 °C for 20 h. The cells were then harvested by centrifugation (10000*g* for 20 min at 4 °C) and washed twice with 100 mM Tris-HCl buffer (pH 6.8). Bacterial pellets were stored at −70 °C. The centrifuged cells were suspended in 10 mL of 1× His-binding buffer (500 mM NaCl, 20 mM Tris, and 5 mM imidazole, pH 6.8) and disrupted by sonication. The lysate was centrifuged at 15000*g* for 20 min to remove the cell debris, and the soluble fraction was filtered through a 0.2 μm pore size filter. The filtrate was loaded on a His-bind resin column (10 mL) equilibrated with 1× His-binding buffer (500 mM NaCl, 20 mM Tris, and 5 mM imidazole, pH 6.8), and the recombinant proteins were eluted by the same buffer with the linear gradient of imidazole (from 5 mM to 1 M). The fractions containing enzyme activity were pooled and dialyzed against 3 L of Tris-HCl buffer (pH 6.8), and dialyzed enzyme preparation was stored at 4 °C and used for enzyme characterization. The protein concentration was determined by use of a Bio-Rad protein assay kit, with bovine serum albumin as a standard (*23*).

Determination of Molecular Mass and Isoelectric Point. The molecular mass of the denatured protein was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). An SDS−12.5% polyacrylamide gel was prepared by the method of Laemmli (*24*). Proteins were stained with Coomassie brilliant blue G. The molecular mass of the enzyme subunit was estimated using protein marker as standards. The molecular mass of the native protein was determined by gel filtration on a Superose 12HR 5/30 column, and γ-globulin (160000 Da), bovine serum albumin (67000 Da), ovalbumin (43000 Da), and carbonic anhydrase (30000 Da) were used as the reference proteins. Isoelectric point (pI) was estimated by PAGE with 6.25% ampholine (pH 3.5−10) in a gel rod (0.5 cm × 10 cm) using a

kit for isoelecric focusing calibration according to recommendations by the supplier.

Enzyme Assay. The β-galactosidase activity was measured by the amount of *o*-nitrophenol released from *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The reaction mixture (1 mL) contained 10 μL (0.13 μg) of enzyme solution and 990 μL of 100 mM Tris-HCl buffer (pH 6.8) containing ONPG at a concentration of 22 mM. The mixture was incubated at 20 °C for 10 min, and the reaction was terminated by adding an equal volume of 1.0 M Na_2CO_3. The released *o*-nitrophenol was quantitatively determined by measuring the A_{420} of the reaction solution. One unit of activity was defined as the amount of enzyme liberating 1 μmol of *o*-nitrophenol per min. The specific activity was expressed as units per milligram of protein. Assays for activity on lactose were carried out in the same buffer, but the reaction was stopped by boiling the sample for 10 min, and the concentration of glucose was determined using a glucose oxidase−peroxidase assay kit (*12*). One unit of enzyme activity was defined as the amount of activity required to release 1 μmol of glucose per minute.

Effect of pH on Enzyme Activity. For the determination of optimum pH of the enzyme, activities were measured for a pH range of 5.0−10.0 in increments of 0.5 pH units under standard assay conditions. The buffers used were 0.1 M sodium acetate−acetic acid (pH 5.0−6.0), 0.1 M phosphate (pH 6.0−8.0), and 0.1 M potassium chloride−boric acid (pH 8.0−10.0).

Effect of Temperature on Enzyme Activity and Thermostability. The optimal temperature of enzyme activity was determined for a temperature range of 0−50 °C for 5 min under standard assay conditions. The thermostability assay was performed by incubating aliquots of enzyme at different temperatures. Aliquots were removed at various times and assayed under standard conditions.

Effect of Metal Ions on Enzyme Activity. The possible requirement for metal ions was examined by first incubating purified enzyme with 100 mM EDTA in Tris-HCl buffer (pH 6.8) for 3 h at 4.0 °C to remove easily bound divalent cations. The enzyme was then applied to a sephadex G-25 column and eluted with 100 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 6.8). MOPS buffer was substituted for Tris-HCl buffer (pH 6.8), which contained sodium ions that interfered with the cation analysis. Equivalent numbers of units of EDTA-treated and non-EDTA-treated enzyme (used as control) were added to 100 mM MOPS buffer (pH 6.8). The EDTA-treated enzyme was measured at 20 °C in the presence of different concentrations of $CuSO_4$, $ZnSO_4$, $MgCl_2$, $CaCl_2$, $MnCl_2$, $NiCl_2$, $CoCl_2$, NaCl, or KCl.

Hydrolysis of Lactose in Milk. Milk containing 5% (w/v) lactose was added to 2.5 μg of the recombinant BgaP or the current commercial *Kluyveromyces marxianus* var. lactis β-galactosidase, and the solution was incubated with shaking (150 rpm) at 5 °C. After definite time intervals, aliquots of the digest were collected and mixed with the same volume of 10% trichloroacetic acid solution and centrifuged, and the supernatant pH was adjusted in the range of 6−7 using NaOH prior to analysis of glucose formed.

Substrate Specificity and Kinetic Studies. The substrate specificity was estimated by incubating the enzyme at 20 °C for 5 min in 100 mM Tris-HCl buffer (pH 6.8) containing 5 mM final concentration of nitrophenyl substrates. Substrates used were ONPG, ρ-nitrophenyl-β-D-galactopyranoside, *o*-nitrophenyl-β-D-fucopyranoside, ρ-nitrophenyl-β-D-mannoside, *o*-nitrophenyl-β-D-glucoside, ρ-nitrophenyl-β-D-xyloside, ρ-nitrophenyl-β-D-cellobioside, ρ-nitrophenyl-β-D-arabinoside, ρ-nitrophenyl-β-D-lactoside, ρ-nitrophenyl-β-D-galacturonide, ρ-nitrophenyl-β-D-glucuronide, and ρ-nitrophenyl-α-D-galactoside. Michaelis−Menten kinetic parameters for activity of purified enzyme were determined from substrate saturation assays by using ONPG ranging from 0.5 to 22 mM or lactose ranging from 1 to 600 mM as the substrates. Values for the maximum velocity and half-saturation coffefficient (K_m) were determined by plotting the substrate concentration vs the initial velocity of each reaction and subjecting the data to nonlinear regression analysis. Kinetic analyses by curve fitting were performed with the SigmaPlot software.

Nucleotide Sequence Accession Numbers. The nucleotide sequence data reported here have been submitted to the nucleotide sequence databases under accession numbers DQ899950 (*bgaP*) and DQ435614 (16S rRNA), respectively.

RESULTS

Characterization of the Psychrotrophic and Halotolerant *Planococcus* sp. L4. The psychrotrophic and halotolerant *Planococcus* sp. L4 was chosen for further study because it grew on lactose and hydrolyzed X-Gal at 2 °C, an indicator of cold-active β-galactosidase activity. The morphological and physiological properties of strain L4 were as follows: Gram staining, motility, and catalase positive; oxidase, urease, phosphatase, nitrate reduction, spore-forming, hydrolysis of aesculin and starch, the indole, methyl red, Voges-Proskauer tests, and levan formation negative; G ± C content, 56 mol %; and cocci-forming shape with 1−1.5 μm in diameter. The orange-pigmented colonies were about 2−3 mm in diameter after 3 days of incubation at 20 °C. Strain L4 could grow between 2 and 36 °C, pH from 5.0 to 10.0 and salt concentrations from 0 to 25%. To determine the phylogenetic position of the isolate, almost the complete 16S rRNA sequence (1518 bp) of strain L4 was determined and deposited at GenBank accession no. DQ435614. Comparing the 16S rRNA sequence of strain L4 with those in the NCBI database suggested that strain L4 exhibited levels of the 16S rDNA identity of 98% to type strains of *Planomicrobium* sp. 3057 (AM111008) and *Planococcus* sp. Tibet-IIVa2 (DQ108396), respectively. Identification of strain L4 as a *Planococcus* isolate is consistent with the physiological properties and habit of organisms since other *Planococcus* strains have been isolated from marine environments and Antarctic sea ice brine (*25*, *26*). According to biological and biochemical characteristics as well as the 16S rDNA gene analysis, the isolate was classified as *Planococcus* sp. and designated as strain L4.

Characterization of Cold-Active β-Galactosidase Gene. The psychrotrophic and halotolerant *Planococcus* sp. L4 chromosomal library was prepared in *E. coli* BL21 (DE3). The plasmid pUC19Δ lacZ was used to construct the library, and ampicillin-resistant transformants were selected and screened for the ability to hydrolyze X-Gal. Among the approximately 13000 transformants, three positive tansformants were selected as blue colonies on plates containing X-Gal at 20 °C. Restriction digests of these inserts showed that they were derived from the same fragment. Sequence data from the shortest construct contained an open reading frame (ORF) encoding a full-length β-galactosidase gene, *bgaP* (**Figure 1**). The β-galactosidase encoding by this ORF contained 677 amino acids, giving a calculated molecular mass of 77.3117 kDa and a theoretical pI of 4.8. The deduced amino acid sequence was used to compare with other amino acid sequences deposed in the NCBI database. The psychrotrophic and halotolerant *Planococcus* sp. L4 β-galactosidase was found to be a member of the family 42 glycosyl hydrolase. The enzyme showed the following percentage identities to β-galactosidases from the following microorganisms: *Planococcus* sp. "SOS Orange" isolate (92%), *Bacillus halodurans* C-125 (55%), *Bacillus circulans* (51%), and *Bacillus clausii* KSM-K16 (47%). There is no signal peptide amino acid sequence in mature protein from the putative start codon to the N-terminal. The G + C content in the coding region is 41%. In addition, structural parameters potentially involved in adaptation to low temperature were compared by using sequences of psychrotrophic and halotolerant *Planococcus* sp. L4 β-galactosidase (BgaP), mesophilic *E. coli* β-galactosidase (LacZ) (*27*), and *Arthrobacter* strain SB β-galactosidase (BgaS) with the lowest optimal temperature (*16*). In cold-active β-galactosidase (BgaP), the content of proline residues (3.8%), which affect the backone flexibility and thus the local mobility of the chain, is

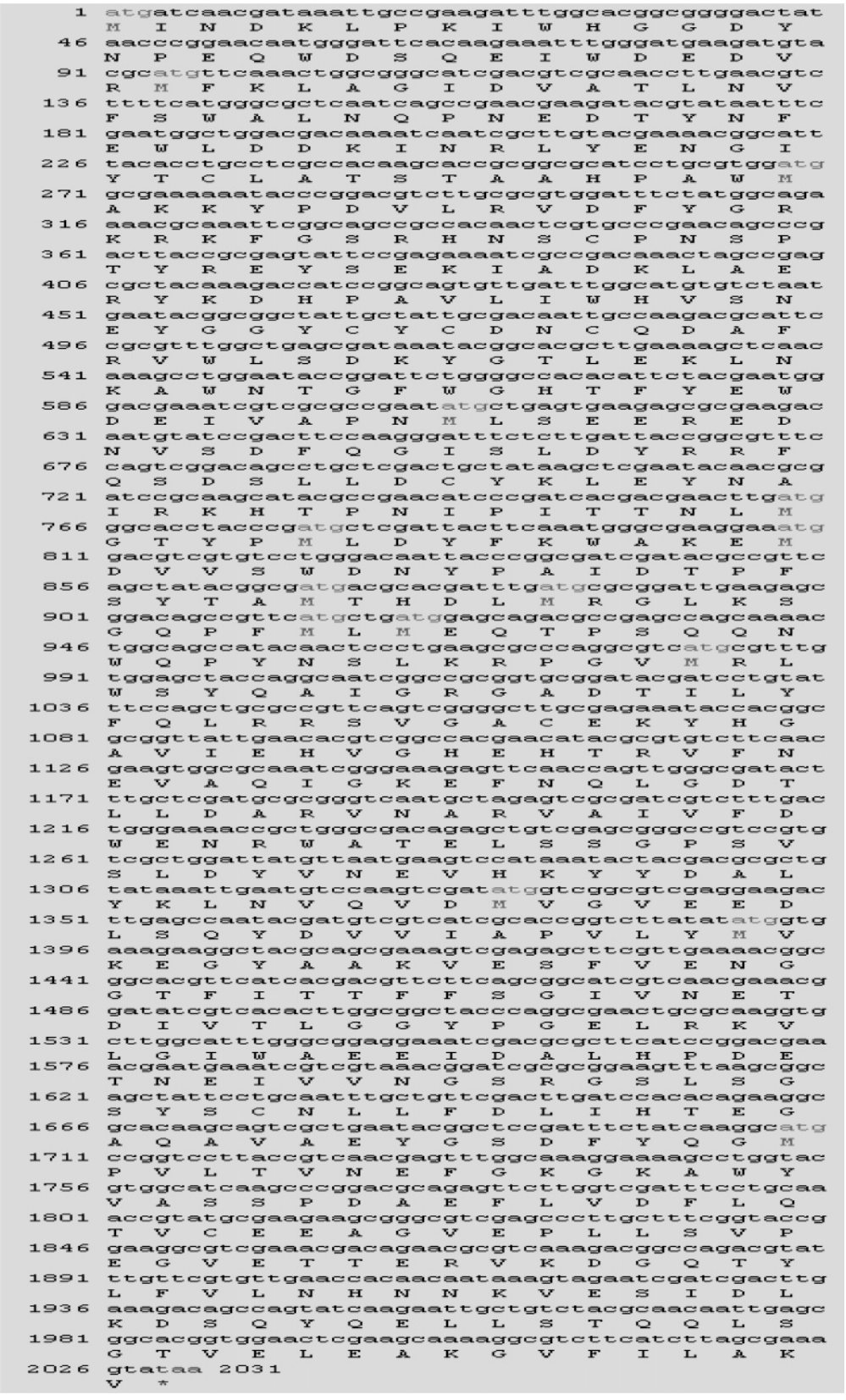

Figure 1. Nucleotide and deduced amino acid sequence of BgaP from *Planococcus* sp. L4.

less than the content of proline residues (6.1%) in *E. coli* β-galactosidase. The content of arginine residues, which have the potential to form multiple ion pairs and H bonds, is less than the content of lysine residues, as shown by the decrease in the Arg/(Arg + Lys) ratio. The pI of BgaP (4.8) is lower than that of *E. coli* β-galactosidase (5.28) due to higher and lower contents of acidic and basic amino acids, respectively, in BgaP. David et al. proposed that a high acidic residue content on a protein surface results in increased interaction between the protein and the solvent, which destabilizes the protein structure (*28*). However, the other structural parameters of BgaP were not consistent with trends thought to confer low-temperature

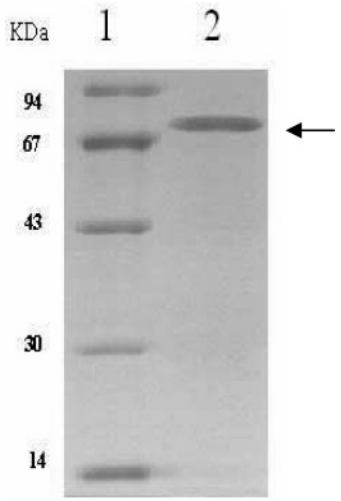

Figure 2. SDS-PAGE analysis of the purified BgaP from *Planococcus* sp. L4 (lane 2) and protein markers (lane 1) stained with Coomassie blue. Markers from top to bottom are phosphorylase b (94000 Da), bovine serum albumin (67000 Da), ovalbumin (43000 Da), carbonic anhydrase (30000 Da), and α-lactalbumin (14000 Da).

activity, such as glycine content, GRAVY (grand average of hydropathicity), and aliphatic index.

Overexpression and Purification of Recombinant BgaP. To investigate the biochemical properties of BgaP, we expressed the BgaP protein with a six-histidine tag at its C terminus in *E. coli* BL21 (DE3) and purified the protein to homogeneity. Bacteria transformed with the expression vector and induced with IPTG abundantly expressed the histidine-tagged protein. The *E. coli* cells were harvested and disrupted by sonification. Cell debris was removed by centrifugation (10000*g* for 20 min at 4 °C). The supernatant was the crude extract of the enzyme, which was then further purified by Ni-NTA-agarose chromatography. The molecular mass of the recombinant protein was about 78000 Da by SDS-PAGE (**Figure 2**), in good agreement with the molecular mass deduced from the nucleotide sequence (77311 Da). The relative molecular mass of native enzyme estimated by gel filtration on a calibrated column of Sephacryl 200 HR was 155000 Da. Hence, it is assumed that the purified enzyme (BgaP) is a dimer. The pI value was estimated to be 4.8. The N-terminal amino acid sequence of the first 15 residues of the recombinant protein was determined to be MEICTKG-SRKHLTSRASE by automated Edman degradation. This was identical to the sequence deduced from the nucleotide sequence. The experimentally determined isoelectric point of recombinant BgaP was pH 4.8. Thus, we successfully overexpressed and purified recombinant BgaP.

Biochemical Characterization of Recombinant BgaP. To determine the optimal pH for recombinant BgaP, we measured the enzyme activity at various pH values (pH 5.0−10.0), using ONPG as a substrate, at 20 °C. BgaP exhibited above 80% of its maximal activity in the pH range of 6.0−8.0, with the highest activity at a pH of approximately 6.8 (**Figure 3**). The thermodependency of activity results showed that the highest specific activity with ONPG was at 20 °C. Lowering or raising the temperature from 20 °C resulted in reduction of β-galactosidase activity. Recombinant BgaP exhibited 27% of the maximal activity even at 0 °C, but enzyme activity decreased with a further increase in temperature until it was undetectable above 50 °C (**Figure 4**). To examine the thermal stability of recombinant BgaP, we incubated the enzyme at a particular temperature and measured its residual activity under standard

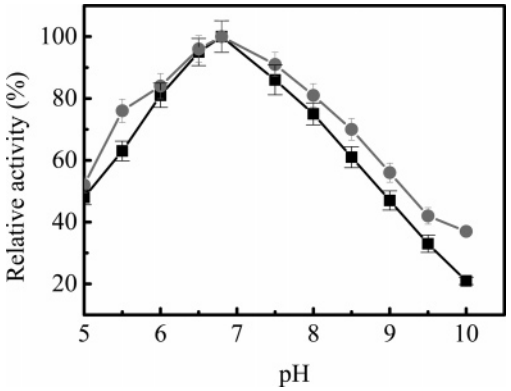

Figure 3. Effect of pH on activity (■) and stability (●) of BgaP from *Planococcus* sp. L4.

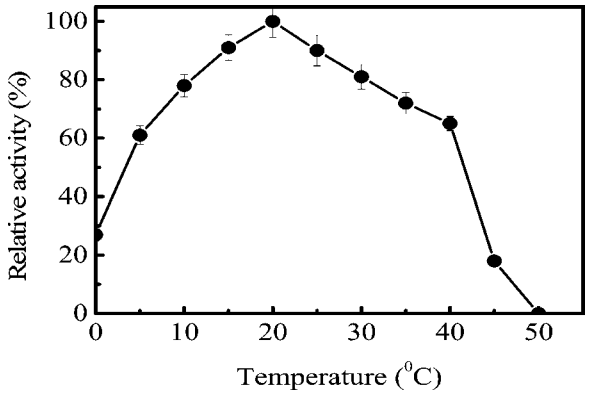

Figure 4. Effect of temperature on activity of BgaP from *Planococcus* sp. L4.

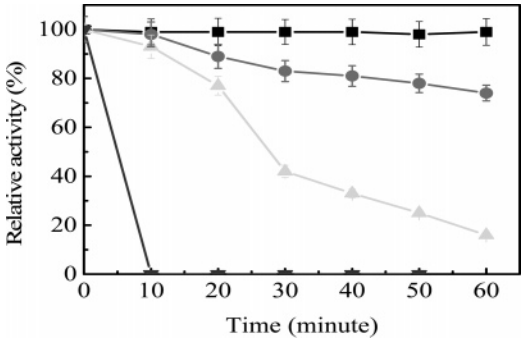

Figure 5. Thermostability of purified BgaP from *Planococcus* sp. L4 at different temperatures; enzyme was incubated at 25 (■), 35 (●), 40 (▲), and 45 °C (▼), and samples were removed at various times and assayed for activity under standard conditions.

assay conditions. It was noted that the recombinant BgaP was rather stable at or below 25 °C but lost 58% activity after 30 min at 40 °C and inactivated after only 10 min at 45 °C (**Figure 5**).

To examine the possible metal ion requirements of the enzyme, the enzyme preparation was treated with EDTA to remove metal ions and desalted by chromatography on Sephadex G-25 column, and the enzyme was eluted from the column with 100 mM MOPS buffer (pH 7.0). No activity was lost during treatment with 100 mM EDTA after 2 h, and the addition of 1 mM $MgCl_2$, 1 mM $CaCl_2$, or 1 mM $MnCl_2$ had no effect; however, the enzyme activity was inhibited by Zn^{2+}, Cu^{2+}, Ni^{2+}, or Co^{2+} to different extents (**Table 1**). However, addition of Na^+ or K^+ slightly stimulated the enzyme activity at low concentrations, and the optimal concentration was 250 mM. A further increase of their concentration of ions above the optimum

Table 1. Effect of the Various Substances on Relative Activity of BgaP from *Planococcus* sp. L4

substances	relative activity (%)
none	100
10 mM $MgCl_2$	99 ± 4.2
10 mM $MnCl_2$	97 ± 4.3
10 mM $CaCl_2$	98 ± 4.7
1 mM $NiCl_2$	53 ± 2.3
1 mM $CuSO_4$	87 ± 4.3
1 mM $ZnSO_4$	83 ± 4.6
1 mM $CoCl_2$	57 ± 2.5
200 mM EDTA	98 ± 4.6

value resulted in a decrease in enzyme activity. The enzyme was still active even in the presence of Na^+ or K^+ at a concentration up to 5 M (**Figure 6**).

A study of the substrate specificity of recombinant BgaP was performed by comparing enzymatic activity on a variety of chromogenic nitrophenyl analogues. Recombinant BgaP displayed the highest level of activity with ONPG as substrate and intermediate level of activity with ρ-nitrophenyl-β-D-galactopyranoside. Lower levels of activity were observed with all other chromogenic nitrophenyl analogues, and activities were less than 0.01% of the ONPG activity.

To further examine the activity of recombinant BgaP, the K_m values were determined at a different temperature with ONPG as the substrate. The highest specific activity (k_{cat}) was 271.7 s^{-1} at 20 °C. The half-saturation coefficient (K_m) was highest at 5 °C and decreased with increasing temperature (2.9 μM at 20 °C). The highest k_{cat}/K_m value (93.7 s^{-1} mM^{-1}) was also observed at 20 °C, and 54.7% of this value was retained at 10 °C. The kinetic parameters for recombinant BgaP were also determined with lactose as the substrate. It had k_{cat}/K_m values of 2.1 and 7.0 at 5 and 20 °C, respectively. Both values are higher than that reported for *E. coli* β-galactosidase (**Table 2**) (*9*). The K_i value for D-galactose (38 mM) was similar to mesophilic *K. lactis* β-galactosidase (42 mM) and significantly higher than those reported for LacZ (10−20 mM) (*29*, *30*).

Experiments on lactose digestion in milk were performed by using identical concentrations of recombinant BgaP and the current commercial *K. lactis* β-galactosidase. The results showed that 36% of milk lactose was hydrolyzed by BgaP and only 14% was removed by *K. lactis* β-galactosidase after 60 min at 5 °C (*9*).

DISCUSSION

The industrial applications of psychrophilic and psychrotrophic microorganisms have been recognized for a long time; the main application of their cold-active enzymes was in food, dairy, detergent, and the environmental industry (*31*). We are especially interested in investigating the properties of cold-active β-galactosidase to gain basic insight into the function of enzymes at low temperature and remove lactose from milk and whey effectively under refrigeration conditions. Although the genetic and biochemical properties of some cold-active β-galactosidases have been reported (*6*−*9*, *11*−*17*), studying novel cold-active β-galactosidase may further provide insight into the evolution of genes and structural relationships, obtain enzyme with excellent properties, and illuminate the unique features responsible for thermal adaptation. The isolate L4 producing β-galactosidase activity was identified as *Planococcus* sp. on the basis of physiological and biochemical characteristics and the 16S rDNA gene analysis. In view of its salt and temperature optima as well as growth conditions, this strain should be regarded as a psychrotolerant and halotolerant bacterium (*32*).

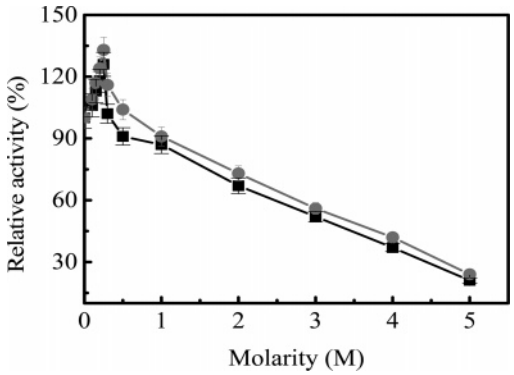

Figure 6. Effect of NaCl or KCl on activity of BgaP from *Planococcus* sp. L4.

Table 2. Kinetic Parameters of BgaP from *Planococcus* sp. L4 for the Hydrolysis of Lactose and ONPG

substrate	temperature (°C)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
ONPG	5	5.4 ± 0.33	174.8 ± 9.2	32.4
ONPG	10	3.8 ± 0.21	195 ± 10.3	51.3
ONPG	20	2.9 ± 0.16	271.7 ± 14.2	93.7
lactose	5	20.4 ± 1.7	43 ± 2.5	2.1
lactose	10	11.2 ± 0.56	62 ± 3.3	5.5
lactose	20	10.4 ± 0.52	73 ± 3.8	7.0

A library of the psychrotrophic and halotolerant *Planococcus* sp. L4 genomic DNA was constructed in pUC19Δ lacZ and screened with X-Gal on LB agar plates at 20 °C. A full-length β-galactosidase gene from the shortest construct was cloned, sequenced, and followed by heterologous expression in *E. coli* and characterization of the individual enzyme. From sequence analysis, it is obvious that recombinant BgaP is a member of family 42 glycosyl hydrolases. The active enzyme is a dimer, and each of its two subunits with molecular mass close to 78 kDa consists of 677 amino acids.

Activity characterization revealed that the optimal temperature for BgaP (20 °C) is similar to that of *Arthrobacter* strain SB β-galactosidase (18 °C) (*16*) and lower than those previously reported many other β-galactosidases (whether they were from psychrotolerant, or psychrophilic bacteria), which generally have been found to be exhibited at optimal temperatures around 30−50 °C (*8*, *12*, *17*). Of special importance for future comparison of cold-active enzymes to their higher-temperature counterparts is the finding that the BgaP protein is most closely related to the *Planococcus* SOS orange β-galactosidase and shows 92% amino acid identity with a difference of only 54 amino acids. The BgaP has an optimal temperature at 20 °C, which is below that of *Planococcus* SOS orange β-galactosidase (22 °C), and the feature is the same as β-galactosidases from *Pseudoalteromonas* species; one β-galactosidase from *P. haloplanktis* had an optimum of about 45 °C (*9*), whereas one from a closely related *Pseudoalteromonas* species had an optimum of 26 °C, with about 28% of its maximal activity remaining at 5 °C (*7*).

The temperature stability of BgaP is greatly lower than that of *E. coli* β-galactosidase. This mesophilic β-galactosidase of *E. coli* has lost only 20% of its activity after 10 min at 45 °C, whereas BgaP was inactivated after only 10 min at 45 °C (*12*). The BgaP exhibited 27% of its activity at 0 °C as compared to *E. coli* β-galactosidase, which is completely inactive around this

temperature (*9*). The pH optimum of the BgaP (pH 6.8) was lower than those recorded from *P. haloplanktis* (pH 8.5) and *E. coli* (8.0) (*9*), higher than that reported from *Pseudoalteromonas* sp. 22b (pH6.0) (*8*), and similar to those previously described *Planococcus* SOS orange β-galactosidase (6.5) (*17*) and *Arthrobacter* strain SB β-galactosidase (7.0) (*16*). EDTA was no inhibitory even at high concentration (100 mM), indicating that metal ions are not required for enzyme function. This observation does not quite agree with data reported by Hoyoux and Coker (*9*, *16*), but the feature is the same as β-galactosidases from *Planococcus* SOS orange (*17*). Na^+ or K^+ at low concentration slightly stimulated the enzyme activity, but it has a high tolerance for salt and still retained 27 and 22% in the presence of 5 M KCl or 5 M NaCl, respectively. In light of this property, application of the BgaP will be more efficient and useful than enzymes from other sources in substrate such as milk, skim, and whey, which contain substantial amounts of Na^+ and K^+. In general, the catalytic efficiencies (k_{cat}/K_m) are considered as a measurement of the enzyme's specificity (*1*). The lowered speed of chemical reaction for cold-active enzymes at low temperature is compensated by the change of kinetic parameters such as an increase of specificity constant. This could be caused by the increase of k_{cat} or contrary by a decrease of K_m or by combination of the changes of both of these parameters (*33*). The catalytic efficiencies of BagP for lactose had 47 times higher than that of *E. coli* β-galactosidase at 20 °C. Moreover, it was able to hydrolyze 34% of milk lactose after 60 min at 5 °C. Thus, these results showed that the BgaP was thermolabile and had a high activity at low temperature.

The amino acid composition analysis was carried out to determine whether trends proposed by others to be characteristic of either cold activity or thermal stability were noted (*2*). The results were not consistent with the suggested trends except fewer pralines or arginines, a lower Arg/(Arg + Lys) ratio, and lower pI, suggesting that these changes conferring thermal adaptation are more subtle and are not reflected in an overall amino acid composition. The same observations were found that trends in amino acid composition do not always reflect thermostability (*16*, *34*). To better determine structural features necessary for or unique to activity at low temperature, a larger database developed with technique that enable direct comparison of proteins with few structural differences is needed (*35*).

In a word, the novel cold-active β-galactosidase gene from the psychrotrophic and halotolerant *Planococcus* sp. L4 was cloned and sequenced, and the recombinant BgaP has been purified and characterized. The β-galactosidase described here differs from those previously reported in at least one of the following aspects: molecular mass, pI, resistance to many metals, pH, and temperature optima (*6*−*9*, *11*−*17*). These differences of the isofunctional enzyme suggest diversity in evolution and a spread of β-galactosidase gene among different microorganisms. Taking into consideration that the ideal β-galactosidase requirements for removing milk lactose would work well (*13*), cold-active β-galactosidase from the psychrotrophic and halotolerant *Planococcus* sp. L4 could conceivably be developed to fulfill the practical requirements to enable its use in food industry due to the combination of low-temperature activity, salt tolerance, and optimal activity at pH 6.8. Further study is helpful to establish more molecular knowledge on gene overexpression and structure−function relationships.

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