Biosci. Biotechnol. Biochem., **70** (2), 495–499, 2006

Molecular Cloning and Characterization of an Enzyme Hydrolyzing *p*-Nitrophenyl α-D-Glucoside from *Bacillus stearothermophilus* SA0301

Atsushi KOBAYASHI,* Takashi TONOZUKA,† Kimihiko SATO, Mikita SUYAMA, Jun SASAKI, Batbold NYAMDAWAA, Masayoshi SAKAGUCHI,** and Yoshiyuki SAKANO

Department of Applied Biological Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

Received September 20, 2005; Accepted October 30, 2005

***Bacillus stearothermophilus* SA0301 produces an extracellular oligo-1,6-glucosidase (bsO16G) that also hydrolyzes *p*-nitrophenyl α-D-glucoside (Tonozuka *et al.*, *J. Appl. Glycosci.*, 45, 397–400 (1998)). We cloned a gene for an enzyme hydrolyzing *p*-nitrophenyl α-D-glucoside, which was different from the one mentioned above, from *B. stearothermophilus* SA0301. The k_0/K_m values of bsO16G for isomaltotriose and isomaltose were 13.2 and 1.39 $s^{-1}\cdot mM^{-1}$ respectively, while the newly cloned enzyme did not hydrolyze isomaltotriose, and the k_0/K_m value for isomaltose was 0.81 $s^{-1}\cdot mM^{-1}$. The primary structure of the cloned enzyme more closely resembled those of trehalose-6-phosphate hydrolases than those of oligo-1,6-glucosidases, and the cloned enzyme hydrolyzed trehalose 6-phosphate. An open reading frame encoding a protein homologous to the trehalose-specific IIBC component of the phopshotransferase system was also found upstream of the gene for this enzyme.**

Key words: oligo-1,6-glucosidase; *p*-nitrophenyl α-D-glucoside; isomaltooligosaccharide; trehalose-6-phosphate hydrolase; *Bacillus stearothermophilus* SA0301

p-Nitrophenyl α-D-glucopyranoside (*p*NPG) is a powerful tool for the study of glucosidases and related enzymes. Enzymes hydrolyzing *p*NPG can be detected easily, since the hydrolyzate, *p*-nitrophenol, is visible in yellow. Several types of enzymes that hydrolyze *p*NPG have been found. In the glycoside hydrolase family 13 (α-amylase family), the following enzymes have been reported: (1) oligo-1,6-glucosidase (dextrin 6-α-glucanohydrolase, EC 3.2.1.10, abbreviated O16G), which hydrolyzes the α-1,6-glucosidic bonds of isomaltooligosaccharides;[1–4] (2) α-glucosidase (EC 3.2.1.20), which hydrolyzes α-1,4-glucosidic bonds and releases D-glucose from the non-reducing end side of the substrate[5,6] and some of the enzymes, such as those from *Bacillus* sp. SAM1606, also show broad substrate specificity and hydrolyze α-1,1-, α-1,3-, α-1,4-, and α-1,6-linked diglucoses as well as sucrose;[7,8] (3) trehalose-6-phosphate hydrolase (α,α′-trehalose-6-phosphate phosphoglucohydrolase, α,α′-phosphotrehalase, EC 3.2.1.93), which hydrolyzes α,α′-trehalose-6-phosphate to produce glucose and glucose-6-phosphate;[9,10] and (4) dextran glucosidase (EC 3.2.1.70), which mainly hydrolyzes α-1,6-glucosidic linkages of dextran.[11] Several researchers have reported that the primary structures of these *p*NPG-hydrolyzing enzymes are homologous.[8,9,12]

Bacillus stearothermophilus SA0301 produces an extracellular O16G (abbreviated bsO16G).[1] We reported previously that bsO16G hydrolyzes isomaltose and isomaltotriose, but does not hydrolyze α,α′-trehalose. This paper explains that *B. stearothermophilus* SA0301 has another gene encoding a *p*NPG-hydrolyzing enzyme, although molecular cloning of the bsO16G gene was originally intended.

Materials and Methods

DNA manipulations, strains, and plasmids. The gene manipulations were carried out based on those of Sambrook *et al.*[13] *Escherichia coli* MV1184 was used as a host strain. A plasmid, pUC118, was obtained from Takara Bio, Ohtsu, Japan. *Bacillus stearothermophilus* SA0301 was prepared as described previously.[1] Colonies were screened for *p*NPG-hydrolyzing activity as described.[1] The DNA sequences of the constructed plasmids were done using an Applied Biosystems 373A DNA sequencer. The nucleotide sequence data have been submitted to the DDBJ/EMBL/GenBank databases (accession no. AB219424).

Preparation of the cloned pNPG-hydrolyzing enzyme. Preparation of the crude recombinant enzyme was carried out as follows: *E. coli* MV1184 harboring

† To whom correspondence should be addressed. Fax: +81-42-367-5705; E-mail: tonozuka@cc.tuat.ac.jp
* Present address: *Graduate School of Engineering, Tohoku University, Aoba 6-6-11-513, Aoba-ku, Sendai, Miyagi 980-8579, Japan*
** Present address: *Department of Applied Chemistry, Kogakuin University, 1-24-2 Nishi-shinjuku, Shinjuku-ku, Tokyo 163-8677, Japan*

pIM04 was grown on 100 ml Luria-Bertani (LB) medium containing ampicillin (50 μg/ml) to A_{600} = 0.6–0.8, and then induced with isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and the incubation was continued overnight. The cells were harvested by centrifugation at 10,000 × *g* for 5 min, resuspended in 40 ml of 10 mM Tris–HCl buffer (pH 7.5), and disrupted by sonication. The supernatant obtained by centrifugation at 10,000 × *g* for 15 min was pooled as a crude bsPNPGH solution. The solution was used for the protein purification and for the evaluation of the ratio of the enzymatic activities for trehalose 6-phosphate and *p*NPG.

Purification of the cloned pNPG-hydrolyzing enzyme. The crude enzyme was dialyzed against 10 mM Tris–HCl buffer (pH 7.5), and applied onto a DEAE-Toyopearl 650S column (1.8 × 16 cm, Tosoh, Tokyo, Japan) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0–0.2 M sodium chloride in the same buffer at a flow rate of 3 ml/min. The active fractions were collected and dialyzed against 10 mM Tris–HCl buffer (pH 7.5), and applied onto a Mono Q HR 5/5 column (0.5 × 5 cm, Amersham). The procedures of equilibration and elution were the same as for the DEAE-Toyopearl 650S column.

Preparation of bsO16G. Preparation and purification of the extracellular enzyme (bsO16G) from *B. stearothermophilus* SA0301 were carried out as described previously.[1)]

Substrates for enzyme assays. Isomaltose (Glc*p*-α-(1→6)-Glc), isomaltotriose (Glc*p*-α-(1→6)-Glc*p*-α-(1→6)-Glc) and palatinose (Glc*p*-α-(1→6)-Fru*f*) were purchased from Wako Pure Chemicals, Osaka, Japan. α,α′-Trehalose (Glc*p*-α-(1→1)-Glc*p*) was obtained from Hayashibara, Okayama, Japan. *p*NPG and trehalose 6-phosphate was purchased from Sigma, St. Louis, MO, USA.

Enzyme and protein assays. The *p*NPG-hydrolyzing activity (for 5 mM *p*NPG in 50 mM McIlvaine buffer, pH 6.5, at 60 °C) was evaluated as described perviously.[1)] One unit of enzymatic activity was defined as the amount of 1 μmol of released *p*-nitrophenol per min. Kinetic parameters (including parameters for *p*NPG) were determined as follows: A reaction mixture (1 ml) containing the substrate, 50 mM McIlvaine buffer (pH 6.5), and bsPNPGH or bsO16G was incubated at 60 °C, and the portions (125 μl) were taken at 5-min intervals to confirm the linearity of the reaction. The reaction was stopped by adding equal amounts of 40 mM sodium hydroxide, and the amount of liberated glucose was measured using glucose-oxidase and peroxidase as described[1,14)] for all the substrates listed in Table 2. For evaluation of the ratio of the enzymatic activities for trehalose 6-phosphate and *p*NPG (5 mM each), 10 mM Tris–HCl buffer (pH 7.0) was used instead of McIlvaine buffer to eliminate the effect of phosphate on the hydrolysis. The activities were calculated from the amount of released glucose (the glucose-oxidase/peroxidase method, for trehalose 6-phosphate) or *p*-nitrophenol (for *p*NPG). Protein concentrations were determined by the method of Lowry *et al.*, as described previously.[14)]

Primary sequence analysis. Similarity searches were performed of the DDBJ database (http://www.ddbj.nig.ac.jp/) using the BLAST program. Alignment of the sequences and their phylogenetic distances were calculated at the DDBJ database using the ClustalW program. The phylogenetic tree was illustrated with the TreeView program.[15)]

Results and Discussion

Molecular cloning of a gene for a pNPG-hydrolyzing enzyme

A genomic DNA of *B. stearothermophilus* SA0301 was prepared and partially digested with *Sau*3AI. After separation using electrophoresis on a 1% agarose gel, fragments of 4 to 10 kb in length were recovered and inserted at the *Bam*HI site of the plasmid pUC118. *E. coli* MV1184 cells were transformed with the recombinant plasmids. Colonies were screened for *p*NPG-hydrolyzing activity and one clone was obtained. The plasmid was designated pIM01 and the physical map was determined (Fig. 1). The 2.2-kb *Kpn*I-*Acc*I fragment of pIM01 was subcloned into pUC118, resulting in plasmid pIM04 (Fig. 1). *p*NPG-hydrolyzing activity was detected in the *E. coli* MV1184 cells harboring pIM04 when 0.5 mM IPTG was present in the medium. It was not clear that the gene encodes the enzyme we initially intended to clone (bsO16G), and hence the newly cloned enzyme was tentatively designated bsPNPGH.

Purification and effect of pH and temperature of recombinant bsPNPGH

E. coli MV1184 harboring pIM04 was cultivated, and recombinant bsPNPGH was purified in two chromatography steps using a DEAE-Toyopearl 650S column and a Mono Q HR 5/5 column (Table 1). SDS–PAGE with Coomassie Brilliant Blue R staining gave a single band, and the molecular mass was found to be approximately 65 kDa (data not shown). The N-terminal amino acid

Table 1. Summary of Purification of Recombinant bsPNPGH

	Total activity (unit)	Yield (%)	Specific activity (unit/mg)	Purification (fold)
Crude enzyme	348	100	12.8	1
DEAE-Toyopearl 650S	178	51	38.8	3.0
Mono Q HR 5/5	112	32	65.9	5.1

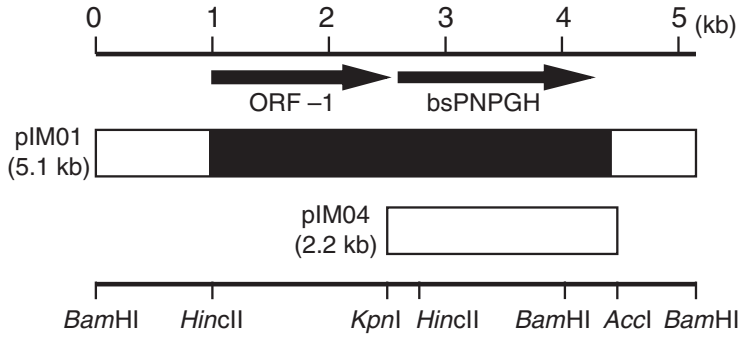

Fig. 1. Physical Maps of Plasmids Carrying the bsPNPGH Gene.

Open reading frames (ORF −1 and bsPNPGH) are shown and the directions are indicated by arrows. The sequenced region in this study (3.3 kb) of pIM01 is printed in black.

Table 2. Kinetic Parameters for Hydrolysis of Various Substrates with bsO16G and bsPNPGH

Substrate	bsO16G			bsPNPGH		
	k_0 (s^{-1})	K_m (mM)	k_0/K_m ($s^{-1}\cdot mM^{-1}$)	k_0 (s^{-1})	K_m (mM)	k_0/K_m ($s^{-1}\cdot mM^{-1}$)
*p*NPG	164 ± 0.23	1.62 ± 0.09	101	52.0 ± 0.21	4.37 ± 0.18	11.9
Isomaltose	5.60 ± 0.07	4.02 ± 0.15	1.39	4.97 ± 0.11	6.15 ± 0.30	0.81
Isomaltotriose	12.6 ± 0.41	0.95 ± 0.06	13.2		ND	
α,α′-Trehalose		ND		0.30 ± 0.03	4.40 ± 0.18	0.068
Palatinose	0.60 ± 0.05	1.34 ± 0.03	0.45	3.90 ± 0.07	4.14 ± 0.28	0.94
Methyl α-D-glucoside	0.20 ± 0.01	5.98 ± 0.53	0.033	0.23 ± 0.01	7.29 ± 0.41	0.032

ND, Activity not detected.

sequence of the recombinant bsPNPGH was analyzed using a Beckman L3000 protein sequencer and determined to be MNKQPWWKK-. We reported previously that the N-terminal amino acid sequence of bsO16G was determined to be MERKWWEA-,[1] which is different from that of recombinant bsPNPGH.

The effect of pH and temperature on bsPNPGH activity was examined using *p*NPG as a substrate. The optimal pH and temperature were pH 5.5–6.5 and 50–60 °C respectively. The enzyme was stable (residual activity > 90%) in the range from 5.5 to 9.5 (at 50 °C for 10 min), and also stable up to 65 °C (at pH 6.5 for 10 min). These values were similar to those for bsO16G.[1]

Comparison of the kinetic parameters of bsPNPGH and bsO16G

The kinetic parameters of bsPNPGH and bsO16G for several saccharides were determined. Both enzymes were unable to hydrolyze maltose. Although both enzymes hydrolyzed *p*NPG, isomaltose, palatinose, and methyl α-D-glucoside, their kinetic parameters were different (Table 2). The k_0/K_m values of bsO16G for *p*NPG and isomaltose (101 and 1.39 $s^{-1}\cdot mM^{-1}$ respectively) were higher than those of bsPNPGH (11.9 and 0.81 $s^{-1}\cdot mM^{-1}$ respectively). The k_0/K_m value of bsO16G for isomaltotriose was higher than that for isomaltose (13.2 and 1.39 $s^{-1}\cdot mM^{-1}$ respectively), and bsO16G did not hydrolyze α,α′-trehalose. These results indicate that the principal substrates of bsO16G are isomaltooligosaccharides. In contrast, bsPNPGH did not hydrolyze isomaltotriose but did hydrolyze α,α′-trehalose. Other than with an artificial substrate, *p*NPG, the k_0/K_m values of bsPNPGH were low, and thus none of the natural sugars listed in Table 2 were found to be the substrate for bsPNPGH.

Comparison of the primary structures of bsPNPGH and related enzymes

The nucleotide sequence of a portion of 3.3 kb of pIM01 (printed in black in Fig. 1) was determined. The open reading frame of bsPNPGH consisted of 1,689 nucleotides starting with an ATG codon, which corresponds to a protein of 563 amino acid residues. The calculated molecular mass was 66 kDa. The deduced N-terminal amino acid sequence of bsPNPGH was identified as MNKQPWWKK-, identical to the N-terminal amino acid sequence determined with the protein sequencer. No sequence for the N-terminal amino acid sequence of bsO16G, MERKWWEA-,[1] was found in the deduced primary structure of bsPNPGH, indicating that the bsPNPGH gene is different from the bsO16G gene we originally intended to clone.

A homology search of the deduced primary structure of bsPNPGH was carried out, and the phylogenetic tree of bsPNPGH and related enzymes is shown in Fig. 2A. bsPNPGH most resembled an α-glucosidase from *Bacillus* sp. DG0303 (TrEMBL no. Q9L872, 88% identity), and also resembled trehalose-6-phosphate hydrolases from several *Bacilli*, such as *Bacillus* sp.

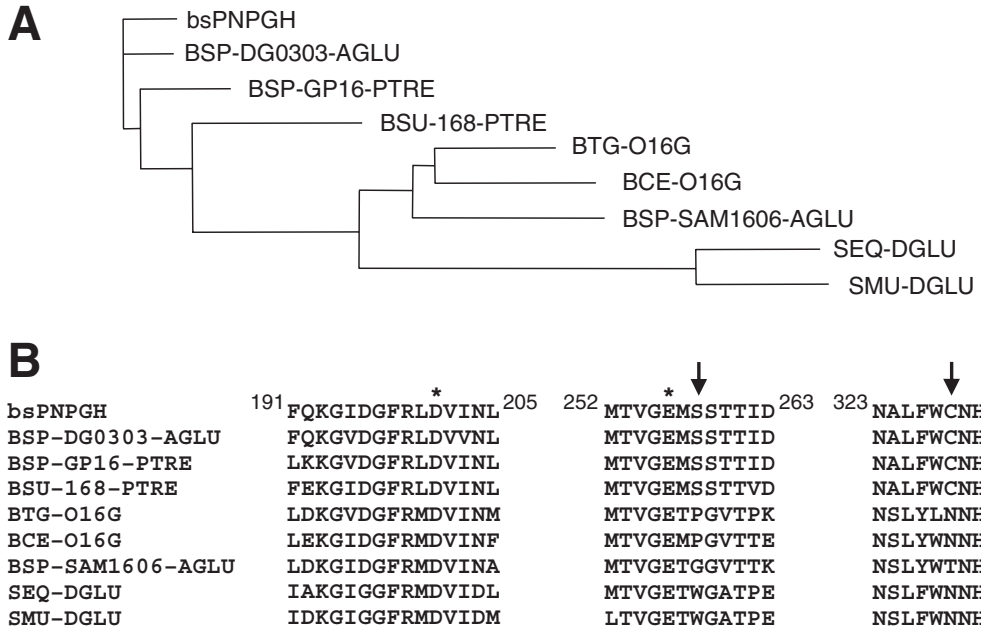

Fig. 2. Comparison of the Primary Structures of bsPNPGH and Related Enzymes.

A, Phylogenetic tree of the enzymes. The tree was created with the programs Clustal W (DDBJ) and TreeView. *Abbreviations*: BSP-DG0303-AGLU, *Bacillus* sp. DG0303 α-glucosidase (TrEMBL Q9L872); BSP-GP16-PTRE, *Bacillus* sp. GP16 trehalose-6-phosphate hydrolase (PRF 2922284A); BSU-168-PTRE, *Bacillus subtilis* trehalose-6-phosphate hydrolase (Swiss-Prot P39795); BTG-O16G, *Bacillus thermoglucosidasius* oligo-1,6-glucosidase (Swiss-Prot P29094); BCE-O16G *Bacillus cereus* oligo-1,6-glucosidase (Swiss-Prot P21332); BSP-SAM1606-AGLU, *Bacillus* sp. SAM1606 α-glucosidase (TrEMBL Q45517); SEQ-DGLU, *Streptococcus equisimilis* dextran glucosidase (Swiss-Prot Q59905); SMU-DGLU, *Streptococcus mutans* dextran glucosidase (Swiss-Prot Q99040). B, Comparison of the amino acid sequences in the vicinity of the catalytic residues. The numbering of the amino acid sequence of bsPNPGH is given. Three catalytic residues of α-amylase family enzymes are indicated by asterisks. Arrows above the sequences indicated the residues that are important for the substrate specificities of the *Bacillus* sp. SAM1606 α-glucosidase proposed by Noguchi *et al.*[8]

GP16 (PRF no. 2922284A, 82% identity) and *Bacillus subtilis* 168[9] (Swiss-Prot no. P39795, 71% identity). bsPNPGH showed homology with O16Gs and dextran glucosidases, although the scores (about 50–60% identity) were lower than those for trehalose-6-phosphate hydrolases (Fig. 2A).

Enzymes belonging to the α-amylase family have three acidic residues, which are identified as catalytic residues.[16,17] Based on the alignment of the primary structures of bsPNPGH, trehalose-6-phosphate hydrolases, O16Gs, and dextran glucosidases, Asp201, Glu256, and Asp331 of bsPNPGH, appear to function as catalytic residues (Fig. 2B). Sequences in the vicinity of the catalytic residues of these enzymes were found to be highly conserved (Fig. 2B). Noguchi *et al.* reported that the replacement of the two amino acid residues indicated by arrows in Fig. 2B of *Bacillus* sp. SAM1606 α-glucosidase significantly altered the substrate specificity.[8] These two residues (Ser258 and Cys328) of bsPNPGH were identical to those of *Bacillus* sp. DG0303 α-glucosidase and trehalose-6-phosphate hydrolases (Fig. 2B).

Nucleotide sequence of an open reading frame located upstream of the bsPNPGH gene

An open reading frame was located 80 bp upstream of the bsPNPGH gene, and was designated ORF -1 (Fig. 1). The ORF -1 gene was transcribed in the same direction as the bsPNPGH gene. The ORF -1 gene consisted of 1,413 nucleotides, starting with an ATG codon, which corresponds to a protein of 471 amino acid residues. The deduced primary structure of ORF -1 was highly homologous to bacillary trehalose-specific IIBC components (about 70–90% identity) of the phosphotransferase system (PTS), the major sugar transport system in many Gram-positive and Gram-negative bacterial species.[18,19]

bsPNPGH hydrolyzed trehalose 6-phosphate

The observations described above strongly suggest that bsPNPGH exhibits the activity of trehalose-6-phosphate hydrolase. To confirm this, a crude bsPNPGH solution was prepared and the ratio of the enzymatic activities for trehalose 6-phosphate and *p*NPG (5 mM each) was determined. The activity for trehalose 6-phosphate (101 $\mu mol \cdot min^{-1} \cdot ml^{-1}$) was 45 times as high as that for *p*NPG (2.26 $\mu mol \cdot min^{-1} \cdot ml^{-1}$). The crude extract from *E. coli* MV1184 harboring pUC118 was also prepared by the same procedures. The extract hydrolyzed neither trehalose 6-phosphate nor *p*NPG under the same conditions. The results suggest that bsPNPGH is a trehalose-6-phosphate hydrolase, and it is reasonable to redesignate it as bsPTRE for future studies.

α-Amylase family enzymes are composed of three common domains, A, B, and C.[17] Janeček *et al.* analyzed the amino acid sequences of domain B of α-amylase family enzymes.[12] Domain B from *Bacillus cereus* O16G and *Streptococcus mutans* dextran glucosidase resemble each other very closely, and the report refers to "domain B of the oligo-1,6-glucosidase type." Interestingly, *S. mutans* dextran glucosidase not only attacks dextran, but also splits *p*NPG, a molecule much smaller than dextran.[11] In this study, a BLAST search for bsPNPGH retrieved O16Gs, α-glucosidases, trehalose-6-phosphate hydrolases, and dextran glucosidases, all of which are known to be *p*NPG-hydrolyzing enzymes. These findings suggest that the primary structures of the enzymes hydrolyzing *p*NPG are homologous, while their enzymatic and physiological properties are quite diverse. We have reported that intracellular and extracellular *p*NPG-hydrolyzing activities were observed in different periods during the cultivation of *B. stearothermophilus* SA0301.[1] The intracellular and the extracelluar activities were detected mainly during the logarithmic and stationary phases respectively. On the basis of the results from kinetic studies and primary structure analysis, it is likely that the intracellular enzyme is to be identified as bsPNPGH, which participates in PTS-mediated metabolism, while the extracelluar enzyme is to be identified as bsO16G, which engages in hydrolyzing isomaltooligosaccharides on the outside of the cell. Although the primary structures of the *p*NPG-hydrolyzing enzymes are homologous to each other, their substrate specificities for natural saccharides are diverse. Our observations suggest that *B. stearothermophilus* produces two *p*NPG-hydrolyzing enzymes whose physiological roles are different. Further studies of these two enzymes might help to elucidate the mechanism of the substrate recognition of the *p*NPG-hydrolyzing enzymes. Oligonucleotides encoding the N-terminal amino acid sequence of bsO16G and also amino acid sequences conserved among O16Gs were prepared, and PCR amplifications were carried out using several combinations of these nucleotides. No clone for bsO16G has been obtained so far, and molecular cloning of bsO16G is now in progress.

References

1) Tonozuka, T., Sato, K., Sakaguchi, M., Suyama, K., Sekine, K., and Sakano, Y., Purification and some properties of an extracellular oligo-1,6-glucosidase from *Bacillus stearothermophilus* SA0301. *J. Appl. Glycosci.*, **45**, 397–400 (1998).
2) Suzuki, Y., and Oishi, K., A relationship between efficiency of isomaltosaccharide hydrolysis and thermostability of six *Bacillus* oligo-1,6-glucosidases. *Appl. Microbiol. Biotechnol.*, **31**, 32–37 (1989).
3) Watanabe, K., and Suzuki, Y., Protein thermostabilization by proline substitution. *J. Mol. Cat. B: Enzymatic*, **4**, 167–180 (1998).
4) Yamamoto, M., and Horikoshi, K., Purification and properties of an oligo-1,6-D-glucosidase from an alkalophilic *Bacillus* species. *Carbohydr. Res.*, **197**, 227–235 (1990).
5) Chiba, S., Molecular mechanism in α-glucosidase and glucoamylase. *Biosci. Biotechnol. Biochem.*, **61**, 1233–1239 (1997).
6) Nishio, T., Hakamata, W., Kimura, A., Chiba, S., Takatsuki, A., Kawachi, R., and Oku, T., Glycon specificity profiling of α-glucosidases using monodeoxy and mono-*O*-methyl derivatives of *p*-nitrophenyl α-D-glucopyranoside. *Carbohydr. Res.*, **337**, 629–634 (2002).
7) Inohara-Ochiai, M., Nakayama, T., Goto, R., Nakao, M., Ueda, T., and Shibano, Y., Altering substrate specificity of *Bacillus* sp. SAM1606 α-glucosidase by comparative site-specific mutagenesis. *J. Biol. Chem.*, **272**, 1601–1607 (1997).
8) Noguchi, A., Yano, M., Ohshima, Y., Hemmi, H., Inohara-Ochiai, M., Okada, M., Min, K.-S., Nakayama, T., and Nishino, T., Deciphering the molecular basis of the broad substrate specificity of α-glucosidase from *Bacillus* sp. SAM1606. *J. Biochem.*, **134**, 543–550 (2003).
9) Gotsche, S., and Dahl, M. K., Purification and characterization of the phospho-α(1,1)glucosidase (TreA) of *Bacillus subtilis* 168. *J. Bacteriol.*, **177**, 2721–2726 (1995).
10) Rimmele, M., and Boos, W., Trehalose-6-phosphate hydrolase of *Escherichia coli*. *J. Bacteriol.*, **176**, 5654–5664 (1994).
11) Russell, R. R. B., and Ferretti, J. J., Nucleotide sequence of the dextran glucosidase (dexB) gene of *Streptococcus mutans*. *J. Gen. Microbiol.*, **136**, 803–810 (1990).
12) Janeček, Š., Svensson, B., and Henrissat, B., Domain evolution in the α-amylase family. *J. Mol. Evol.*, **45**, 322–331 (1997).
13) Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989).
14) Tonozuka, T., Mogi, S., Shimura, Y., Ibuka, A., Sakai, H., Matsuzawa, H., Sakano, Y., and Ohta, T., Comparison of primary structures and substrate specificities of two pullulan-hydrolyzing α-amylases, TVA I and TVA II, from *Thermoactinomyces vulgaris* R-47. *Biochim. Biophys. Acta*, **1252**, 35–42 (1995).
15) Page, R. D. M., TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.*, **12**, 357–358 (1996).
16) Kuriki, T., and Imanaka, T., The concept of the α-amylase family: structural similarity and common catalytic mechanism. *J. Biosci. Bioeng.*, **87**, 557–565 (1999).
17) MacGregor, E. A., Janeček, Š., and Svensson, B., Relationship of structure to specificity in the α-amylase family of enzymes. *Biochim. Biophys. Acta*, **1546**, 1–20 (2001).
18) Plumbridge, J., Regulation of gene expression in the PTS in *Escherichia coli*: the role and interactions of Mlc. *Curr. Opin. Microbiol.*, **5**, 187–193 (2002).
19) Siebold, C., Flükiger, K., Beutler, R., and Erni, B., Carbohydrate transporters of the bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS). *FEBS Lett.*, **504**, 104–111 (2001).