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

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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 k₀/Kₘ values of bsO16G for isomaltotriose and isomaltose were 13.2 and 1.39 s⁻¹·mM⁻¹, respectively, while the newly cloned enzyme did not hydrolyze isomaltotriose, and the k₀/Kₘ value for isomaltose was 0.81 s⁻¹·mM⁻¹. The primary structure of the cloned enzyme more closely resembled those of trehalose-6-phosphate hydrolyses 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 phosphotransferase 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 (pNPG) is a powerful tool for the study of glucosidases and related enzymes. Enzymes hydrolyzing pNPG can be detected easily, since the hydrolyzate, p-nitrophenol, is visible in yellow. Several types of enzymes that hydrolyze pNPG have been found. In the glucoside 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 substrate⁵⁻⁶ 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’-phosphothrehalase, 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 pNPG-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 pNPG-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 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

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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 pNPG.

**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 (Glcα-(1→6)-Glc), isomaltotriose (Glcα-(1→6)-Glcα-(1→6)-Glc) and palatinose (Glcα-(1→6)-Fru) were purchased from Wako Pure Chemicals, Osaka, Japan. α,α′-Trehalose (Glcα-(1→1)-Glcα) was obtained from Hayashibara, Okayama, Japan. pNPG and trehalose 6-phosphate was purchased from Sigma, St. Louis, MO, USA.

**Enzyme and protein assays.** The pNPG-hydrolyzing activity (for 5 mM pNPG in 50 mM McIlvaine buffer, pH 6.5, at 60°C) was evaluated as described previously.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 pNPG) 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 described1,14 for all the substrates listed in Table 2. For evaluation of the ratio of the enzymatic activities for trehalose 6-phosphate and pNPG (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 pNPG). Protein concentrations were determined by the method of Lowry *et al.*, as described previously.1,14

**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.1,15

**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 Sau3AI. After separation using electrophoresis on a 1% agarose gel, fragments of 4 to 10 kb in length were recovered and inserted at the BamHI site of the plasmid pUC118. *E. coli* MV1184 cells were transformed with the recombinant plasmids. Colonies were screened for pNPG-hydrolyzing activity and one clone was obtained. The plasmid was designated pIM01 and the physical map was determined (Fig. 1). The 2.2-kb KpnI-AccI fragment of pIM01 was subcloned into pUC118, resulting in plasmid pIM04 (Fig. 1). pNPG-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**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (unit)</th>
<th>Yield (%)</th>
<th>Specific activity (unit/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>348</td>
<td>100</td>
<td>12.8</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650S</td>
<td>178</td>
<td>51</td>
<td>38.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Mono Q HR 5/5</td>
<td>112</td>
<td>32</td>
<td>65.9</td>
<td>5.1</td>
</tr>
</tbody>
</table>
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-, identical to the N-terminal amino acid sequence of bsPNPGH, indicating that the bsPNPGH gene is different from the bsO16G gene we originally intended to clone.

The effect of pH and temperature on bsPNPGH activity was examined using pNPG 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.

Comparison of the primary structures 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 pNPG, isomaltose, palatinose, and methyl α-D-glucoside, their kinetic parameters were different (Table 2). The $k_0/K_m$ values of bsO16G for pNPG and isomaltose (101 and 1.39 s$^{-1}$·mm$^{-1}$ respectively) were higher than those of bsPNPGH (11.9 and 1.81 s$^{-1}$·mm$^{-1}$ respectively). The $k_0/K_m$ value of bsO16G for isomaltotriose was higher than that for isomaltooligosaccharides. In contrast, bsPNPGH did not hydrolyze isomaltotriose but did hydrolyze α,α'-trehalose. Other than with an artificial substrate, pNPG, 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 plM01 (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 bsPNPGH was carried out, and the phylogenetic tree 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 trehalase-6-phosphate hydrolases from several Bacilli, such as Bacillus sp.
GP16 (PRF no. 292284A, 82% identity) and Bacillus subtilis 168 (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 transferase 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 pNPG (5 mM each) was determined. The activity for trehalose 6-phosphate (101 μmol·min⁻¹·ml⁻¹) was 45 times as high as that for pNPG (2.26 μmol·min⁻¹·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 pNPG 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 pNPG, a molecule much smaller than dextran.¹¹ In this study, a BLAST search for bsPNPG retrieved O16Gs, α-glucosidases, trehalose-6-phosphate hydrolases, and dextran glucosidases, all of which are known to be pNPG-hydrolyzing enzymes. These findings suggest that the primary structures of the enzymes hydrolyzing pNPG are homologous, while their enzymatic and physiological properties are quite diverse. We have reported that intracellular and extracellular pNPG-hydrolyzing activities were observed in different periods during the cultivation of B. stea-thermophilus SA0301.¹ The intracellular and the extracellular 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 bsPNPG, which participates in PTS-mediated metabolism, while the extracellular enzyme is to be identified as bsO16G, which engages in hydrolyzing isomaltotrioligosaccharides on the outside of the cell. Although the primary structures of the pNPG-hydrolyzing enzymes are homologous to each other, their substrate specificities for natural saccharides are diverse. Our observations suggest that B. stea-thermophilus produces two pNPG-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 pNPG-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