Enzymatic mechanism of BglA-1 from *Streptococcus pneumoniae* TIGR4

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Abstract

The 6-phospho-β-glucosidase can hydrolyze 6-phospho-β-glucoside to produce glucose and glucose 6-phosphate (G6P), both of which can enter the energy-generating glycolytic pathway. The 478-residue protein, 6-phospho-β-glucosidase BglA-1 from *S. pneumoniae* TIGR4 is encoded by the gene SP0303, which resides in an eight-gene operon composed by a lactose-type phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) which includes three separate subunits: EIIB (SP0305), EIIA (SP0308), EIIC (SP0310) and a multidomain transcriptional regulator. Here, through enzymatic analysis and sequence alignment, we present the substrate specificity of BglA-1. Glu\(^{\text{432}}\) and Glu\(^{\text{438}}\) as the nucleophile group and the proton donor respectively. Three residues, Phe\(^{\text{131}}\), Tyr\(^{\text{313}}\) and Trp\(^{\text{349}}\) play important roles in the substrate specificity towards the β-(1,4)-linked glucose 6-phosphate (G6P) bond. In addition, Ser\(^{\text{432}}\), Lys\(^{\text{439}}\) and Tyr\(^{\text{440}}\) participate in enzyme discrimination between the hydrolysis of phosphorylated and the non-phosphorylated substrates.

Introduction

*Streptococcus pneumoniae* (*S. pneumoniae*), the important pathogenic microorganism with high mortality rates, is one of the leading causes of acute pneumonia, otitis media, meningitis, and many other serious diseases in humans [1-3]. Pneumococcal invasions lead to more than 1.6 million infections worldwide every year. In developed and developing countries, the treatment and prevention of diseases caused by *S. pneumoniae* still have great burden [4,5]. *S. pneumoniae* can colonize on the human nasopharyngeal mucosa when the baby is born just a few months. The microorganism need abundant energy sources, such as carton sources, to satisfy its growth in host cells during infection and colonization. There are 32 carbohydrates identified as energy sources in *S. pneumoniae*, including three-carbon molecules (glycerol), nine hexoses or hexose derivatives, three α-galactosides, two β-galactosides, four α-glucosides, seven β-glucosides, and six polysaccharides [6]. Carbohydrates, as the important carbon source of *S. pneumoniae*, its accumulation is facilitated by various sugar transporters, including phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) transporters, cation/proton-coupled transporters and ATP-binding cassette (ABC) transporters [7-17]. PEP-PTS systems which is the most ubiquitous transporters in bacteria, transport various carbohydrates, such as glucose, sucrose, mannitol, fructose, lactose, mannose, and cellulose, the β-1,4 disaccharides of D-glucose [18]. The PEP-PTS systems are composed of three components: enzyme I (EI), heat-stable phosphocarrier protein (HPr), and enzyme II (EI) [13,19]. EI and HPr are nonspecific energy-coupling components. EI is consisting of three or four functional subunits (EIIA, EIB, EIIIC, and sometimes EIID) [20]. The transmembrane permeases, EIIIC and EIID, which are responsible for recognition and binding of specific sugar substrates, transport sugars across the cell membrane into the cytoplasm. The sugar molecule is subsequently transferred to EIIA and EIIIB domains and phosphorylated [10]. The PEP-PTS systems transport carbohydrates cointaneously with phosphorylation of C6 (11). The 6-phospho-β-glucosidase BglA-1 from *S. pneumoniae* belongs to glycosidase hydrolase family 1 (GH-1) which possesses a

variety of glycosidyl hydrolases. Some 6-phospho-β-glucosidases are grouped in the glycosidase families GH-4, in addition to GH-1, GH-1 family belongs to a superfamily (or clan) termed GH-A, all members of which possess a catalytic domain of (α/β)\(_h\) TIM barrel fold. Members of GH-1, such as 6-phospho-β-glucosidase (EC 3.2.1.86), 6-phospho-β-galactosidase (EC 3.2.1.85) and β-glucosidase (EC 3.2.1.21) have different substrates specificity (see the CAZy database) [21]. The 6-phospho-β-glucosidase can hydrolyze 6-phospho-β-glucoside to produce glucose and glucose 6-phosphate (G6P), both of which can enter the energy-generating glycolytic pathway [22]. The 478-residue protein, 6-phospho-β-glucosidase BglA-1 from *S. pneumoniae* TIGR4 is encoded by the gene SP0303 which resides in an eight-gene operon (Figure 1A). The operon composed by a lactose-type PTS which includes three separate subunits: EIIB (SP0305), EIIA (SP0308), EIIC (SP0310) and a multidomain transcriptional regulator (Figure 1B) [23]. The primary sequence of BglA-1 is homologous to the following GH-1 enzymes of known structure: *Lactococcus lactis* PGALase, Bacillus polymyxa BglA, Human hCBG and *S. pneumoniae* BglA-2 [24-28]. The structure-based catalytic mechanism of GH-1 Pgalase from *Lactococcus lactis* has been elucidated [24,25]. As reported previouslytwo conserved residues (glutamate or aspartate) function as catalytic residues, one as a nucleophile group and the other as a proton donor. The process of catalysis is as follow: the glycosidic oxygen of the substrate is protonated by the proton donor and forms the transient oxocarbonium state. Then nucleophilic residue attacks the protonated glycosidic bond, with the formation of a glycosyl-enzyme intermediate. Finally, the protonated glycosidic bond is broken by a proton provided

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Key words: 6-phospho-β-glucosidase, phosphoenolpyruvate (PEP)-dependent phosphotransferase system, substrate specificity

Received: February 07, 2018; Accepted: February 23, 2018; Published: February 26, 2018
by a water molecule, forming the product and returning the enzyme to its original protonated state [29,30].

Previously, we have elucidated the mechanism of the substrate specificity towards the (1,4)-linked glucose 6-phosphate (G6P) bond and the hydrolytic selectivity toward phosphorylated rather than non-phosphorylated compounds of 6-phospho-β-glucosidase BglA-2 from *S. pneumoniae* TIGR4 [28]. Here, through enzymatic analysis and sequence alignment, we present the substrate specificity of BglA-1 from *S. pneumoniae* TIGR4. Glu375 and Glu176 function as the nucleophile and the proton donor respectively. Three residues, Phe131, Tyr313, and Tyr349 play important roles in the substrate specificity towards the β (1,4)-linked glucose 6-phosphate (G6P) bond. In addition, Ser312, Lys438, and Tyr440 participate in enzyme discrimination by the hydrolysis of phosphorylated and the non-phosphorylated substrates.

**Experimental procedures**

**Cloning and expression of BglA-1 and its mutants**—The coding region of the *bglA-1* gene was amplified from the genomic DNA of *S. pneumoniae* TIGR4. The *bglA-1* gene and its mutants were respectively constructed into a pET28a-derived expression vector with N-terminal hexahistidine (6×His) tags. Both the wild-type and mutants were overexpressed in *Escherichia coli* (E. coli) strain Rosetta (DE3) (Novagen) using 2×YT culture medium (5 g of NaCl, 16 g of Bacto-Tryptone, and 10 g of yeast extract/liter). The transformed cells were grown at 37°C in the buffer containing 50 mM Na2HPO4/50 mM NaCl. Fractions containing the target protein were collected and loaded onto a nickel–nitrotolyltriacetic acid column (GE Healthcare) equilibrated with the buffer containing (20 mM Tris–Cl, pH 7.5, 100 mM NaCl). Fractions containing the target protein were combined and stored in the same manner as the wild-type protein.

**Purification of BglA-1 and its mutants**—The cells were collected by centrifugation at 4000 x g for 15 min and resuspended in 50 ml of lysis buffer (20 mM Tris–Cl, pH 7.5, 100 mM NaCl). After 5 min of sonication and 25 min of centrifugation at 12,000 x g, the supernatant containing the target protein was collected and loaded onto a nickel–nitrotolyltriacetic acid column (GE Healthcare) equilibrated with the binding buffer (20 mM Tris–Cl, pH 7.5, 100 mM NaCl). The target protein was washed with the binding buffer (20 mM Tris–Cl, pH 7.5, 100 mM NaCl) and eluted with the same buffer containing 250 mM imidazole. Then the target protein was loaded onto a Superdex 200 column (GE Healthcare) equilibrated with the buffer containing (20 mM Tris–Cl, pH 7.5, 100 mM NaCl). Fractions containing the target protein were combined and collected. Samples for enzymatic activity assays were collected at the highest peak fractions without concentration. The purity of protein was assessed by SDS–PAGE, and the protein sample was stored at -80°C.

**Site-directed mutagenesis**—Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the plasmid encoding the wild-type BglA-1 as the template. The mutant proteins were expressed, purified, and stored in the same manner as the wild-type protein.

**Dynamic light scattering**—Dynamic light scattering was measured through a DYNAPRO-99 (Wyatt Technology Corp, 6300 Hollister) using a 532 nm green laser. The sample was measured in single-use UV-plastic cuvettes (Wyatt Technology Corp, 6300 Hollister). Firstly, a time scale of the scattered light intensity fluctuations was measured. Secondly, the instrument was equilibrated for 2 min at 25°C. At last, the molecular weight of the solution protein BglA-1 was analyzed with the software Dynamic V6 (Wyatt Technology Corp, 6300 Hollister).

**Enzymatic activity assays**—The enzymatic parameters of wild-type BglA-1 and its mutants were measured with the substrate p-nitrophenyl-β-D-glucopyranoside-6-phosphate (pNPβGlc6P) following the previous processes with minor changes [31]. All of the reactions were performed at 37°C in the buffer containing 50 mM Na2HPO4/50 mM NaH2PO4, pH 7.5 and triggered by the addition of BglA-1. Using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA), the change in absorption at 405 nm was monitored continuously. The reaction product PNP was calculated according to a standard curve of p-nitrophenol, which was described by Prag et al. The final Michaelis–Menten parameters (Vmax and Km) of BglA-1 were extracted from these data by nonlinear fitting to the Michaelis–Menten equation using the programOrigin 7.5.

**Preparation of 1-Phenyl-3-methyl-5-pyrazolone (PMP) derivatives of saccharides**—PMP derivatization of saccharides was implemented as described previously with minor changes [32–34]. Briefly, the 10 μl reaction mixture was mixed with 10 μl of 0.3 M aqueous NaOH and a 10 μl 0.5 M methanol solution of PMP. The total reaction system of 30 μl mixture was placed to react for 30 min at 70°C.

![Figure 1A](image-url) Genetic organization of the β-glucoside operon in *S. pneumoniae* TIGR4. SP numbers represent the order of gene loci. Values in parentheses are calculated molecular weights of encoded proteins. B. translocation and phosphorylation of cellulose by the multicomponent β-glucoside PEP-PTS and BglA-1-catalyzed hydrolysis of cellulose-6-P

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Wei-Li Y (2018) Enzymatic mechanism of BglA-1 from *Streptococcus pneumoniae* TIGR4. Dig Sys, 2018 doi: 10.15761/DSJ.1000117
were first isolated by Ba^{2+} and ethanol precipitation and further
of the disaccharides with ATP-dependent β-glucoside kinase (BglK, previously [35]. In brief, phosphorylation was effected by incubation
the glucose moiety in these O-β-linked disaccharides was as described
Phosphorylation of the primary hydroxyl groups of the non-reducing
was processed as described previously for equilibration of the column
(Eclipse XDB-C18 column, 4.6×150 mm; Agilent) and separation
of the components at a flow rate of 1 ml/min [34]. Retention times
monosaccharides were determined by comparison with standard
monosaccharide solutions. Kinetic parameters determinations through
three independent experiments were made to calculate the means and
standard deviations for the $K_m$ and $k_{cat}$ values.

Preparation of Cellobiose-6’P, Thiocellobiose-6’P, and pNPβGlc6P-Cellobiose was obtained from Pfanschtiel Laboratories, and thiocellobiose was purchased from Toronto Research Chemicals. Phosphorylation of the primary hydroxyl groups of the non-reducing glucose moiety in these O-β-linked disaccharides was as described previously [35]. In brief, phosphorylation was effected by incubation of the disaccharides with ATP-dependent β-glucoside kinase (BglK, EC 2.7.1.85) from Klebsiella pneumoniae. Phosphorylated derivatives were first isolated by Ba^{2+} and ethanol precipitation and further purified by ion exchange and paper chromatography. Structures and product purity were confirmed by thin layer chromatography, mass spectrometry, and NMR spectroscopy. Chromogenic pNPβGlc6P was prepared by phosphorylation of the C6 hydroxyl moiety of pNPβ-D-glucopyranoside with phosphorus oxychloride in trimethyl phosphate containing a small amount of water [34].

Results and discussion

The state of BglA-1 in the solution-The plasmid of BglA-1 was constructed and overexpressed as described as the experimental procedures. The protein was purified though Ni-chelating affinity chromatography and size exclusion chromatography. And the state of BglA-1 in the solution-the plasmid of BglA-1 was constructed and overexpressed as described as the experimental procedures. The protein was purified though Ni-chelating affinity chromatography without concentration. The purity of protein was assessed by SDS-PAGE. Firstly, we tested enzymatic activity of BglA-1 for other substrates (gentiobiose-6’P or maltose-6’P) (Figure 4). No activity was showed towards these substrates. These results demonstrated the specificity of BglA-1 towards cellobiose-6’P.

Key residues to the substrate specificity towards +1 site of cellobiose-6’P-Sequence alignment results revealed that three residues Phe^{131}, Trp^{313} and Trp^{349} of BglA-1 (corresponding to Tyr^{126}, Trp^{338} and Trp^{349} in BglA-2) were generally conserved (Fig. 3). To identify the putative roles of Phe^{131}, Trp^{313} and Trp^{349} of BglA-1, enzymatic assays towards the site-directed mutants were carried out. The mutant F131A completely lost its activity whereas F131Y retained the activity of approximately 81% compared to the wild-type (Table 1). The results suggested that Tyr^{313} is indispensable for the activity by contributing to the hydrophobic pocket. In addition, the mutant W349A also absolutely lost its activity. These results clearly demonstrated that residues Phe^{131}, Tyr^{313} and Trp^{349} play an important role in the substrate binding and thus might determine the specificity of +1 site sugar.

Key residues to the substrate specificity towards the phosphate group of cellobiose-6’P-As described previously, the phosphate-group binding residues Ser^{432}, Lys^{438} and Tyr^{440} in BglA-2 discriminated the phosphorylated from the non-phosphorylated sugar [34]. Compared between the sequences of BglA-1 and BglA-2, the residues Ser^{432}, Lys^{438} and Tyr^{440} of BglA-1 were highly conserved, corresponding to Ser^{444}, Lys^{450} and Tyr^{442} of BglA-2. Sequence analysis suggested that the three residues are conserved in GH-1 6-phospho-β-glucosidases from bacteria to protozoan as described previously [28]. As demonstrated previously, the residues were substituted by non-polar residues in other GH-1 glycosidases with non-phosphorylated substrate, such as β-glucosidase A from Bacillus polymyxa [26]. To verify the roles of the three residues,
Figure 3. Sequence alignment of BglA-2 and BglA-1. Two conserved catalytic glutamate residues are marked with red triangle. The subsite +1 residues and the phosphate-binding residues are depicted by green and black triangles, respectively.
Figure 4. Enzymatic assays of wild-type BglA-1 and mutants towards gentiobiose-6’P (A) and maltose-6’P (B)

Table 1. Kinetic constants of BglA-1 and mutants towards pNpβGlc6P and cellobiose-6’p

| Substrate | pNpβGlc6P | Cellobiose-6’p |
|-----------|-----------|----------------|
|           | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$μM$^{-1}$) | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$μM$^{-1}$) |
| Enzyme    |           |                 |                                |             |                   |                                |
| Wild Type | 654±4.8   | 136±6.4         | 2.1×10$^{-4}$±4×10$^{-2}$     | 1924±8.9    | 152±3.8           | 7.9×10$^{-2}$±6×10$^{-3}$     |
| E375A     | ND        | ND              | ND                             | ND          | ND                | ND                             |
| E375Q     | ND        | ND              | ND                             | ND          | ND                | ND                             |
| F131A     | ND        | ND              | ND                             | ND          | ND                | ND                             |
| F131Y     | 838±4.6   | 157±6.8         | 0.19±0.1                       | 2102±12.3   | 129±9.8           | 6×10$^{-2}$±4×10$^{-2}$       |
| Y313A     | ND        | ND              | ND                             | ND          | ND                | ND                             |
| Y313F     | ND        | ND              | ND                             | ND          | ND                | ND                             |
| W349A     | ND        | ND              | ND                             | ND          | ND                | ND                             |
| W349A     | ND        | ND              | ND                             | ND          | ND                | ND                             |
| Y440F     | ND        | ND              | ND                             | ND          | ND                | ND                             |
we tested the enzymatic activities towards phosphorylated substrate through a variety of mutants. The results indicate that S432A, K438A and Y440F completely abolished the activities, suggesting the key roles of Ser432, Lys438 and Tyr440 in determining the phosphorylated substrate (Table 1).

Sequence alignment indicated that two catalytic residues Glu176 and Glu276, the +1 site residues, Phe431, Trp433 and Trp439 and phosphate-binding residues, Ser452, Lys468 and Tyr470, were exclusively conserved. The results indicated that BglA-1 might adopt a similar overall structure and hydrolyze the similar substrate as other 6-phospho-β-glucosidase.

In summary, based on sequence alignment analysis and enzymatic assays, we not only elucidated the specificity towards +1 site sugar, but also the specificity towards -1 site phosphate group of 6-phospho-β-glucosides. The results expand our understanding on the substrate recognition pattern and specificity of GH-1 enzymes.

Acknowledgements

This work was supported by the Anhui Provincial Natural Science Foundation (Grant No. 1508085Q49), the school fund project of Anhui Medical University (Grant No. 2015XJK031), the doctoral research fund project of the Second Affiliated Hospital of Anhui Medical University (Grant No. 2014BKJ034) and Natural Science Foundation of Anhui Provincial Education Department (KJ2017A183).

Conflict of interest disclosure

The authors declare that they have no conflict of interest.

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