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

Wei-Li Yu, Xiang Yang, Zhong-Hua Lu, Xiao-Ping Geng and Yun Sun*

Department of Intensive Care Unit, The Second Affiliated Hospital of Anhui Medical University, Hefei, Anhui, 230601, China

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^{375} and Glu^{176} function as the nucleophile group and the proton donor respectively. Three residues, Phe^{131}, Tyr^{313} and Trp^{349} play important roles in the substrate specificity towards the β (1,4)-linked glucose 6-phosphate (G6P) bond. In addition, Ser^{432}, Lys^{438} and Tyr^{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 cellobiose, 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 (EII) [13,19]. EI and HPr are nonspecific energy-coupling components. EII is consisting of three or four functional subunits (EIIA, EIIB, EIIC, and sometimes EIID) [20]. The transmembrane permeases, EIIC 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 EIIB domains and phosphorylated [10]. The PEP-PTS systems transport carbohydrates coinstantaneously with phosphorylation of C6 (11). The 6-phospho-β-glucosidase BglA-1 from *S. pneumoniae* belongs to glycoside hydrolase family 1 (GH-1) which possesses a

varity of glycosyl 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 $(α/β)_8$ 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 previously two 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 oxocarbenium 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
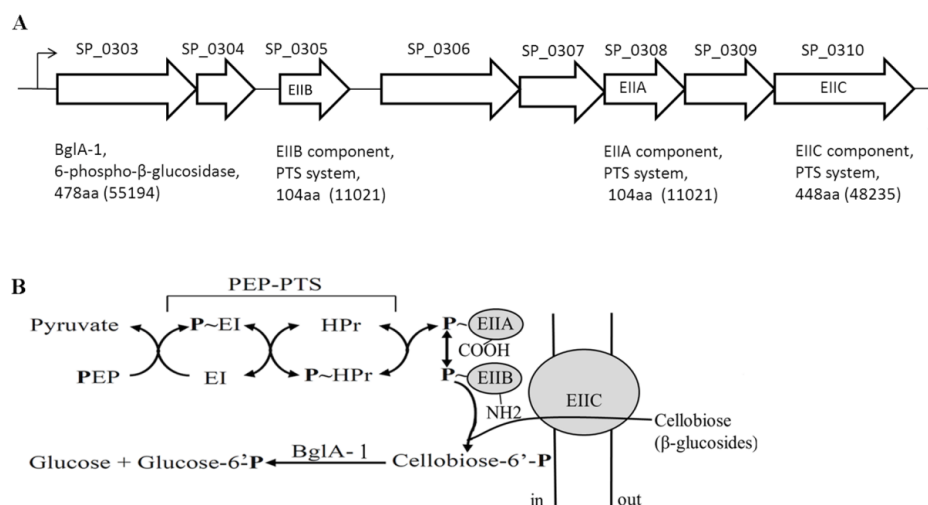
Figure 1A. genetic organization of the β-glucoside operon in *S. pneumonia* TIGR4. SP numbers represent the order of gene loci. Values in parentheses are calculated molecular weights of encoded proteins. **B.** translocation and phosphorylation of cellobiose by the multicomponent β-glucoside PEP-PTS and BglA-1-catalyzed hydrolysis of cellobiose-6′P

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 β (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. Glu[375] and Glu[176] function as the nucleophile group and the proton donor respectively. Three residues, Phe[131], Tyr[313] and Trp[349] play important roles in the substrate specificity towards the β (1,4)-linked glucose 6-phosphate (G6P) bond. In addition, Ser[432], Lys[438] and Tyr[440] participate in enzyme discrimination between 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 2×YT medium containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol. When the OD_{600nm} reached about 0.6 0.8, the recombinant proteins were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 20 h at 16°C.

Purification of BglA-1 and its mutants—The cells were collected by centrifugation at 4000×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×g, the supernatant containing the target protein was collected and loaded onto a nickel-nitrilotriacetic 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 was performed using the QuikChange 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 Na_2HPO_4 /50 mM NaH_2PO_4, 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 (V_{max} and K_m) of BglA-1 were extracted from these data by nonlinear fitting to the Michaelis-Menten equation using the program Origin 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,

then cooled to room temperature, and neutralized with 10 μl of 0.3 M HCl. The creating solution was dissolved in 100 μl of chloroform. After vigorous shaking and centrifuging, the organic phase in the lower was carefully abandoned to remove the excess reagents. The extraction procedure was repeated three times. At last, the aqueous phase containing derivatives was diluted with 40 μl of water before HPLC analysis.

HPLC analysis-The assays towards specific substrate were performed at 37°C in a 10 μl system containing the buffer of 50 mM Na_2HPO_4/50 mM NaH_2PO_4, pH 7.5 and the disaccharide substrate cellobiose-6p′ with kinds of concentrations. The reactions were triggered by the addition of the purified enzymes and terminated by mixing with 10 μl of 0.3 M NaOH. After PMP derivation as mentioned above, the reaction product was centrifuged at 12,000×g for 10 min, and 15 μl supernatant was analyzed by HPLC system (Agilent 1200 Series). Glucose and G6P standards were quantified by HPLC analysis using different concentrations ranging from 0.1 to 1 mM. The mixing buffer composed of 20% acetonitrile and 100 mM Na_2HPO_4/NaH_2PO_4, pH 7.0 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 of 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 Pfanstiehl 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 pNP-β-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 dynamic light scattering was carried out to confirm the state of BglA-1 in the solution. The results of size exclusion chromatography (Figure 2) and dynamic light scattering confirmed the existence of BglA-1 as a dimer in solution.

The enzymatic activity of BglA-1-The samples for enzymatic activity assays were collected at the highest peak fractions of size exclusion chromatography without concentration. The purity of protein was assessed by SDS–PAGE. Firstly, we tested enzymatic activity of BglA-1 for generic substrate (pNPβGlc6P) and specific substrate (cellobiose-6′p). The K_m and k_{cat} values for generic substrate were 654±4.8 μM and 136±6.4 s⁻¹, respectively, and for specific substrate, 1924±8.9 μM and 152±3.8 s⁻¹ (Table 1). These results suggested BglA-1 was a 6-phospho-β-glucosidase. Compared with other GH-1
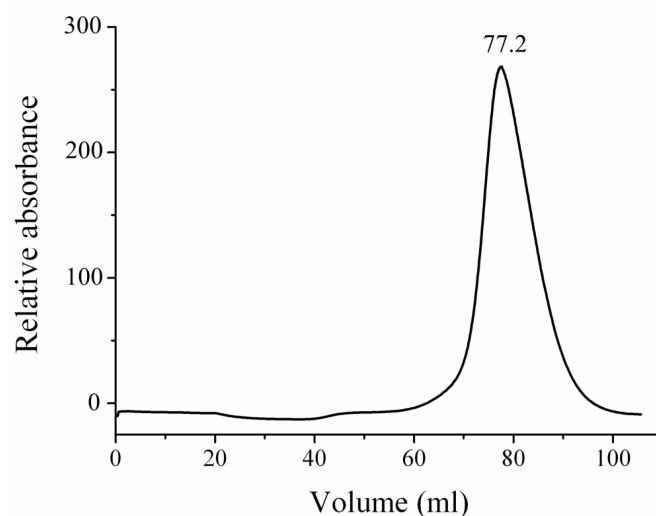

Figure 2. Gel-filtration chromatography of wild-type BglA-1 using Superdex 200 column. The chromogram represents the absorbance at 280 nm. The elution volume in miniliter of the peak was labeled on the top

members of known structures, the active-site residues of BglA-1 are highly conserved, especially two catalytic residues Glu¹⁷⁶ and Glu³⁷⁵ (corresponding to Glu¹⁷¹ and Glu³⁶⁴ in BglA-2) (Figure 3). This suggests that BglA-1 presents a same catalytic mechanism as the previous known structures of GH-1 members [28,29]. The mutants E176A, E176Q, E375A and E375Q completely lost their activities towards pNpβGlc6P and cellobiose-6′p, suggesting the critical roles of Glu¹⁷⁶ and Glu³⁷⁵ of BglA-1 in catalysis (Table 1).

We further tested the enzymatic activity of the wild-type and mutant proteins 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-6p′-Sequence alignment results revealed that three residues Phe¹³¹, Trp³¹³ and Trp³⁴⁹ of BglA-1 (corresponding to Tyr¹²⁶, Trp³⁰³ and Trp³³⁸ in BglA-2) were generally conserved (Fig. 3). To identify the putative roles of Phe¹³¹, Trp³¹³ and Trp³⁴⁹ 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 Tyr³¹³ 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 Phe¹³¹, Tyr³¹³ and Trp³⁴⁹ 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-binding residues Ser⁴²⁴, Lys⁴³⁰ and Tyr⁴³² in BglA-2 discriminated the phosphorylated from the non-phosphorylated sugar [34]. Compared between the sequences of BglA-1 and BglA-2, the residues Ser⁴³², Lys⁴³⁸ and Tyr⁴⁴⁰ of BglA-1 were highly conserved, corresponding to Ser⁴²⁴, Lys⁴³⁰ and Tyr⁴³² of BglA-2. Sequence analysis suggested that the three residues are conserved in GH-1 6-phospho-β-glucosidases from bacteria to protozoon 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,
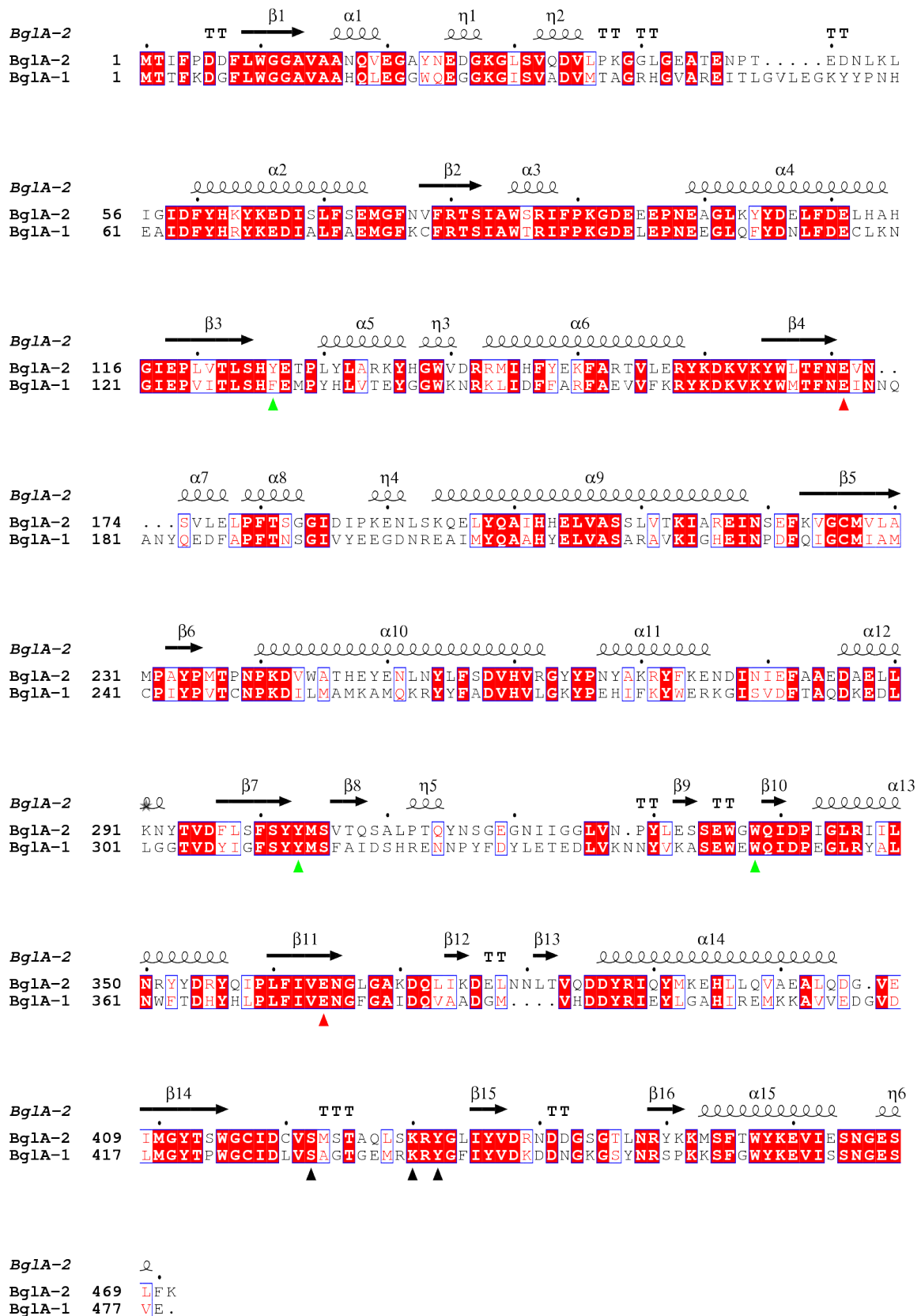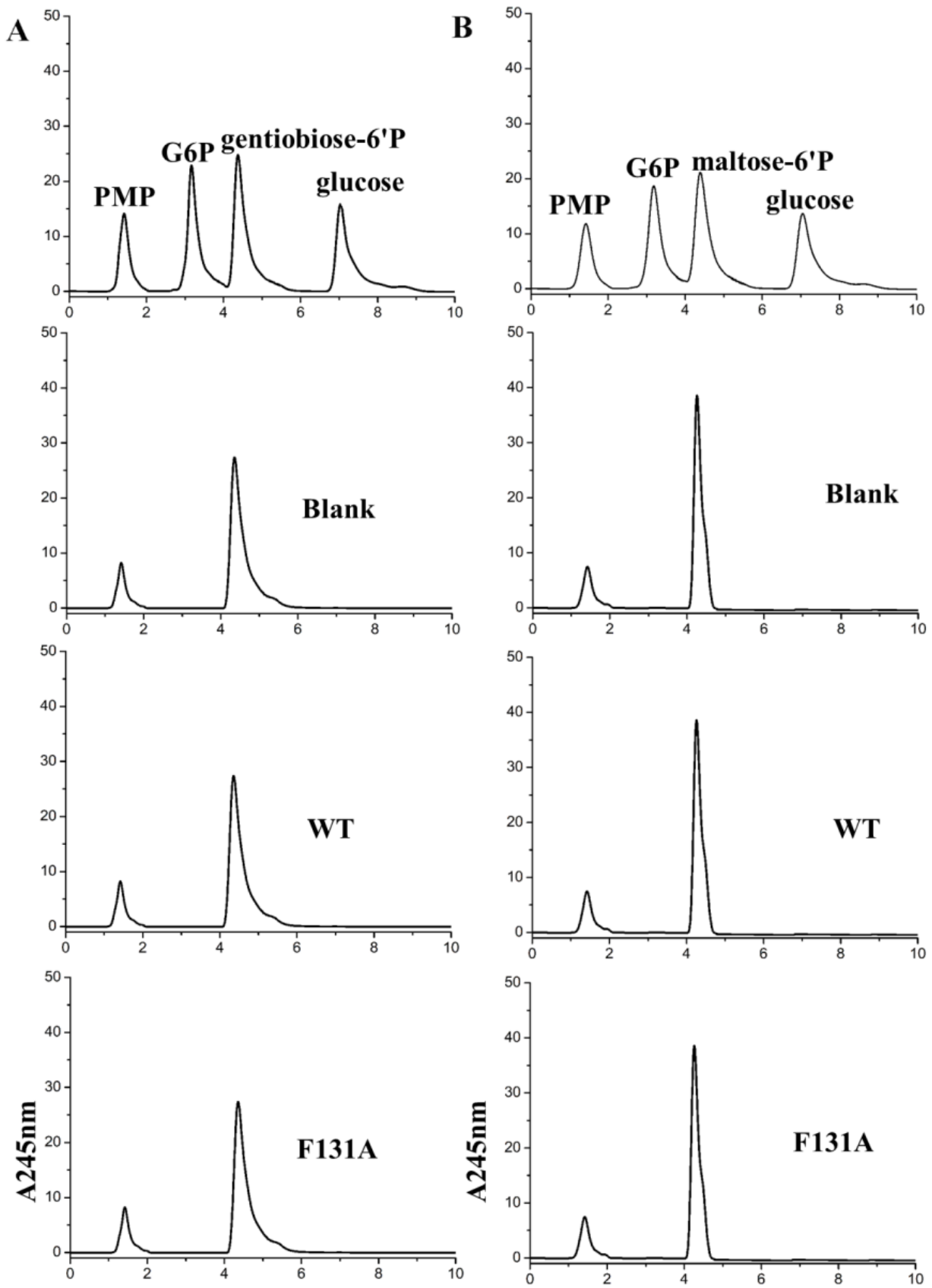
Figure 3. Sequence alignment of BglA-2 and BglA-1. Two conserved catalytic glutamate residues are marked with red triangle. The subsite +1 residues and the phosphate-binding residues are depicted by green and black triangles, respectively
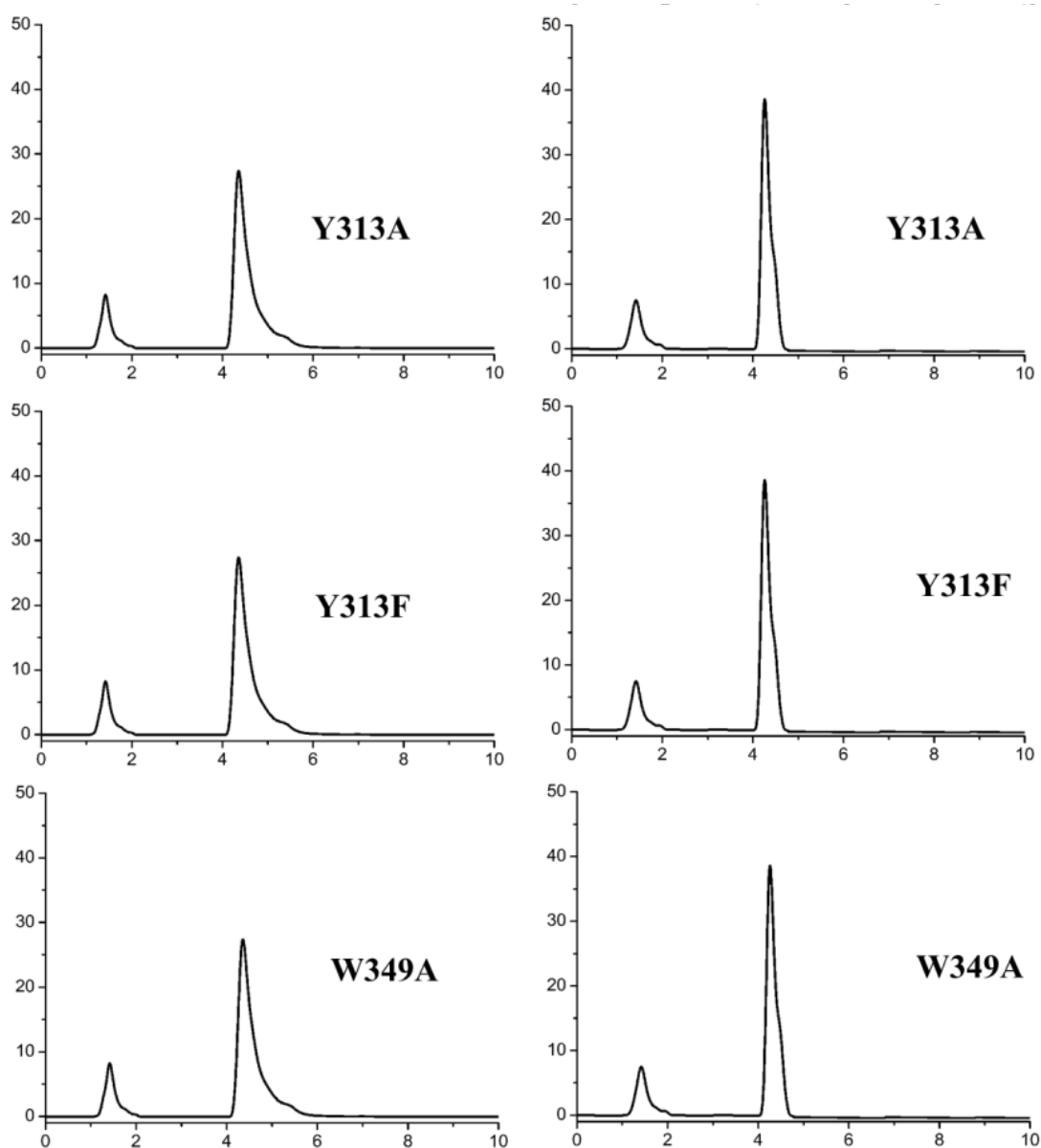
Figure 4. Enzymatic assays of wild-type BglA-1 and mutants towards gentiobiose-6'P (A) and maltose-6'P (B)

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

Substrate	pNpβGlc6P			Cellobiose-6′p		
Enzyme	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})
Wild Type	654±4.8	136±6.4	2.1×10^{-1}±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^{-3}
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 Ser[432], Lys[438] and Tyr[440] in determining the phosphorylated substrate (Table 1).

Sequence alignment indicated that two catalytic residues Glu[176] and Glu[375], the +1 site residues, Phe[131], Trp[313] and Trp[349] and phosphate-binding residues, Ser[432], Lys[438] and Tyr[440], 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 glucose, 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

Conflict of interest disclosure

The authors declare that they have no conflict of interest.

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