Carbohydrate Research 435 (2016) 106–112

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

On the phosphorylase activity of GH3 enzymes: A β-*N*-acetylglucosaminidase from *Herbaspirillum seropedicae* SmR1 and a glucosidase from *Saccharopolyspora erythraea*

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ARTICLE INFO

Article history:
Received 2 August 2016
Received in revised form
21 September 2016
Accepted 21 September 2016
Available online 23 September 2016

Chemical compounds studied in this article:
4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (PubChem CID:102416)
4-nitrophenyl β-D-glucopyranoside (PubChem CID:92930)
β-D-glucopyranosyl phosphate (PubChem CID:122250)
2,4-Dinitrophenyl 2-fluoro-2-deoxy-beta-D-glucopyranoside (PubChem CID:445227)
2-acetamido-2-deoxy-α-D-glucopyranosyl fluoride (PubChem CID:53906163)

Keywords:
Glycoside hydrolase
Glycosidase
GH3 *N*-acetylglucosaminidases
Phospho-sugar
Herbaspirillum seropedicae SmR1
Chemical compounds

ABSTRACT

A phosphorolytic activity has been reported for beta-*N*-acetylglucosaminidases from glycoside hydrolase family 3 (GH3) giving an interesting explanation for an unusual histidine as catalytic acid/base residue and suggesting that members from this family may be phosphorylases [J. Biol. Chem. 2015, 290, 4887]. Here, we describe the characterization of Hsero1941, a GH3 beta-*N*-acetylglucosaminidase from the endophytic nitrogen-fixing bacterium *Herbaspirillum seropedicae* SmR1. The enzyme has significantly higher activity against pNP-beta-D-GlcNAcp (K_m = 0.24 mM, k_{cat} = 1.2 s^{-1}, k_{cat}/K_m = 5.0 $mM^{-1}s^{-1}$) than pNP-beta-D-Glcp (K_m = 33 mM, k_{cat} = 3.3 × 10^{-3} s^{-1}, k_{cat}/K_m = 9 × 10^{-4} $mM^{-1}s^{-1}$). The presence of phosphate failed to significantly modify the kinetic parameters of the reaction. The enzyme showed a broad aglycone site specificity, being able to hydrolyze sugar phosphates beta-D-GlcNAc 1P and beta-D-Glc 1P, albeit at a fraction of the rate of hydrolysis of aryl glycosides. GH3 beta-glucosidase EryBI, that does not have a histidine as the general acid/base residue, also hydrolyzed beta-D-Glc 1P, at comparable rates to Hsero1941. These data indicate that Hsero1941 functions primarily as a hydrolase and that phosphorolytic activity is likely adventitious. The prevalence of histidine as a general acid/base residue is not predictive, nor correlative, with GH3 beta-*N*-acetylglucosaminidases having phosphorolytic activity.

1. Introduction

Glycosidases are enzymes involved in carbohydrate metabolism with broad distribution in all living organisms. These enzymes have attracted considerable attention due to their application as protein targets for drug design [1] and catalysis [2], being utilized not only as catalysts for the depolymerization of complex glycans [3] but also for the synthesis of important targets in glycobiology [4]. Glycosidases catalyze the cleavage of glycosidic bonds, producing an acetal or hemiacetal product through primarily either a retaining or inverting mechanism. A great number of glycosidases show a classical mechanism involving a general acid/base catalysis with Asp or Glu acting as catalytic residues [5], although elegant and alternative strategies have also been reported [6]. According to the amino acid sequence similarity, these enzymes are classified into 135 families in the carbohydrate-active enzymes database (CAZy) [7]. One of the important members in this classification system is glycoside hydrolase family 3 (GH3). This family harbors exo-acting

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http://dx.doi.org/10.1016/j.carres.2016.09.015
0008-6215/

enzymes found in plants, fungi and bacteria that are responsible for assimilation or modification of glycosides and recycling of cell wall components. These enzymes show broad substrate specificity against β-D-glucosides (EC 3.2.1.21), β-D-xylosides (EC 3.2.1.37), α-L-arabinosides (EC 3.2.1.55) and *N*-acetyl-β-D-glucosaminides (EC 3.2.1.52) [8].

In particular, the *N*-acetylglucosaminidases from GH3 are important catalysts in cell wall recycling in Gram-positive and Gram-negative bacteria. In addition, they are responsible for the production of metabolic intermediates involved in the regulation of β-lactamase expression [9,10]. Therefore, these glycosidases are important candidates for design of inhibitors in biomedicinal chemistry [11,12]. In the retaining mechanism proposed for the catalysis, a conserved aspartate acts as a nucleophile to produce a glycosyl-enzyme intermediate, while a histidine residue serves as the acid/base residue [13–15]. Recently, a phosphorolytic activity has been reported for the β-*N*-acetylglucosaminidase produced by *Cellulomonas fimi*, giving an interesting explanation for the unusual histidine in the catalytic site of GH3 members [16]. The presence of the imidazole ring instead of carboxyl groups could avoid charge repulsion with phosphate, making it possible that the enzyme works as a phosphorylase. This discovery has raised the possibility that all GH3 *N*-acetylglucosaminidases may not be hydrolases and additionally suggesting βGlcNAc 1*P* as a new metabolic intermediate in bacterial cell wall metabolism. This phosphate sugar would require at least one enzyme to connect it to the metabolic pathway, and therefore, β-phosphoglucomutases have been suggested as candidates for this task [16].

Although several GH3 β-*N*-acetylglucosaminidases have been characterized [14,16–20], phosphorolysis activity was not considered and consequently this activity has not been monitored. Thus, we decided to characterize a putative GH3 *N*-acetyl glucosaminidase (Hsero1941) from *Herbaspirillum seropedicae* SmR1, in order to investigate its phosphorolytic activity. This organism is an endophytic nitrogen-fixing bacterium that can be found in symbiosis with important commercial crops, such as corn, rice, sugar-cane and wheat and for this reason raises a biotechnological interest [21]. Hsero1941 is a good candidate for this study because according to UniProt database [22], *H. seropedicae* SmR1 codifies only putative phosphoglucomutases (EC 5.4.2.2, EC 5.4.2.8, EC 5.4.2.10) with specificity for α-sugar phosphates. Further, the bacterial plant colonization seems to involve bacterial envelope alterations [23], therefore, characterizing enzymes involved in this process will provide insight into this symbiotic relationship.

2. Results

2.1. Gene cloning and hydrolytic enzymatic activity of Hsero1941

H. seropedicae SmR1 has 23 genes codifying glycoside hydrolases in its genome, according to CAZy analysis, and two of them are classified as enzymes from family 3 (GH3). The *Hsero1941* gene codifies a putative *N*-acetyl-β-D-glucosaminidase with 342 amino acids (M.W. = 36.2 kDa). Sequence alignment with crystallized β-*N*-acetylglucosaminidases from GH3 produced by Gram negative and Gram positive bacteria showed high similarity, including the conserved consensus motif containing the catalytic acid/base histidine (H186) residue (Fig. S1) [13]. The potentially nucleophilic aspartate (D255) residue was also identified. Therefore, we amplified *Hsero1941* from genomic DNA by PCR and cloned it into a pET24b vector. Sequence analysis and partial digestion using restriction endonucleases confirmed the glycosidase gene insertion into the plasmid. The enzyme was expressed with a *C*-terminal His_6-tag to facilitate the purification by affinity chromatography (Fig. S2).

We analyzed the enzyme activity using twelve commercial glycosides (Table S1). The assay indicated a higher activity for *p*NP-β-D-GlcNAc*p* than *p*NP-β-D-GalNAc*p*, together with slight levels of hydrolysis against *p*NP-β-D-Gal*p* and *p*NP-β-D-Glc*p*. These data were also confirmed by determination of steady state kinetic parameters in 50 mM Hepes buffer (Table 1). The k_{cat}/K_m ratio for the GlcNAc glycoside was four orders of magnitude and 500 fold higher than Glc and GalNAc, respectively, which indicated a significant preference for the amino sugar with Glc configuration as substrate. The kinetic parameters for *p*NP-β-D-GalNAc*p* and *p*NP-β-D-Gal*p* were not determined due to the low solubility and slow hydrolytic rate, respectively. The pH effect over Hsero1941 activity was investigated using *p*NP-β-D-GlcNAc*p* as substrate over a pH range of 4.0–9.0 (Fig. S3). The graph showed a wide bell-shaped curve with optimum activity at pH 7.1, which suggests two ionizable amino acid residues in the catalytic site.

2.2. Investigation of phosphorolytic activity of Hsero1941

In order to investigate any potential phosphorolytic activity for Hsero1941, we determined the enzyme activity at different phosphate concentrations (Table 1). This activity is represented by glycosyl phosphorylases, enzymes that catalyze the cleavage of a glycosidic bond through the transfer of a glycosyl unit to inorganic phosphate producing a glycosyl phosphate product [24]. The K_m and k_{cat} values were similar in all assayed conditions, although consistent decreases in k_{cat}/K_m were observed, as phosphate concentrations increased. Together, this data suggests that phosphate has no significant effect on enzyme activity. We analyzed by ^{1}H NMR spectroscopy the cleavage products obtained from incubation of Hsero1941 and *p*NP-β-D-GlcNAc*p* **1** in the presence of phosphate (Fig. 1). We only observed the anomeric signals corresponding to GlcNAc **2** at 5.19 ($J_{1,2}$ = 3.4 Hz, $H1_\alpha$) and 4.70 ppm ($J_{1,2}$ = 7.8 Hz, $H1_\beta$). By monitoring the hydrolysis by Hsero1941 of *p*NP-β-D-GlcNAc*p* in the NMR tube, the beta anomer was observed initially, and upon mutarotation, the alpha isomer was subsequently observed (Fig. S4). These data showed that Hsero1941 has a hydrolytic activity mediated by a double displacement retaining mechanism, which is consistent with the formation of a glycosyl-enzyme intermediate [13].

We decided to investigate whether phosphate, or other anions, could act as nucleophiles in order to cleave the covalently bound intermediate. To this end, Hsero1941 was incubated with DNP2FGlc (**4**), which functions as a covalent inhibitor, trapping the nucleophilic amino acid residue to produce a stable glycosyl-enzyme intermediate [25] (Fig. 2). The inhibition was not complete after 16 h of incubation, since approximately 20% remaining activity was observed. After removal of the excess fluoro glycoside inhibitor, the enzyme was incubated with different anions and the residual activity was evaluated. All anions tested promoted reactivation, with azide (2 M) showing the highest rate of reactivation, almost 2-fold greater than the inhibited enzyme (Fig. 2).

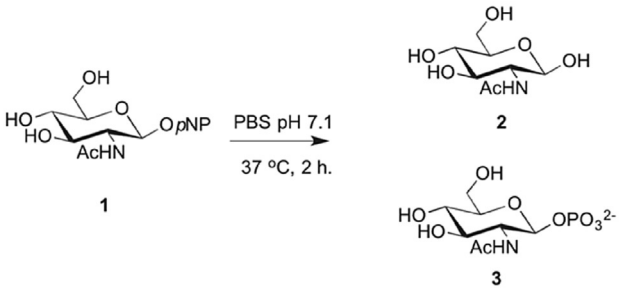
2.3. Hydrolytic activity against glycosyl phosphates catalyzed by Hsero1941, EryBI and DesR

To determine if Hsero1941 was able to hydrolyze a glycosyl phosphate linkage, we incubated commercial βGlc 1*P* (**5**) with Hsero1941, and two known β-glucosidases from GH3: DesR [26] and EryBI [27]. The reaction products were analyzed by ^{1}H NMR spectroscopy (Fig. 3). At all three enzyme concentrations evaluated for Hsero1941, signals corresponding to the anomeric protons of Glc (**6**) at 5.19 and 4.60 ppm were observed indicating that Hsero1941 catalyzed the hydrolysis of **5**. However, EryBI also showed comparable activity at the highest concentration evaluated,

Table 1
Kinetic parameters for substrate hydrolysis by Hsero1941.

Substrate	Phosphate (mM)[a]	K_m (mM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($mM^{-1}s^{-1}$)
*p*NP-β-D-GlcNAc*p*	0	0.24 ± 0.03	1.2 ± 0.10	5.0
*p*NP-β-D-GlcNAc*p*	25	0.29 ± 0.01	1.4 ± 0.03	4.9
*p*NP-β-D-GlcNAc*p*	50	0.30 ± 0.02	1.2 ± 0.02	4.1
*p*NP-β-D-GlcNAc*p*	100	0.27 ± 0.02	1.1 ± 0.01	3.9
*p*NP-β-D-GlcNAc*p*	200	0.33 ± 0.04	1.0 ± 0.10	3.0
*p*NP-β-D-Glc*p*	0	33 ± 4	$3.3 \times 10^{-3} \pm 3 \times 10^{-4}$	9×10^{-4}
*p*NP-β-D-GalNAc*p*	0	ND[b]	ND[b]	0.010

[a] The reactions were performed in 50 mM Hepes pH 7.1 in absence or presence of phosphate. Results are expressed as mean ± standard error of the mean (SEM).
[b] The parameters were not determined due to the linear relationship between V_o and S_o.



Entry	1 (mM)	Hsero1941 (μM)	PBS (mM)	Product[a]
1	5.4	4	10	**2**
2	5.4	4	25	**2**
3	5.4	4	50	**2**
4	5.4	4	100	**2**
5	5.4	4	500	**2**
6	5.4	160	500	**2**
7	10	160	100	**2**

[a] Product was detected by ^{1}H NMR analysis

Fig. 1. Activity of Hsero1941 against *p*NP-β-D-GlcNAc*p* (**1**) at different phosphate concentrations.

demonstrating that it was also able to hydrolyze βGlc 1*P*. In order to evaluate if the hydrolysis of **5** could be a non-specific, or contaminating, activity, Hsero1941 was incubated with *p*NP-phosphate. However, no hydrolytic activity was observed. Together, these data indicated that both Hsero1941 and EryBI are able to hydrolyze **5** at comparable rates.

At this stage, we explored whether βGlcNAc 1*P* (**3**) could be synthesized from a fluoride glycosyl donor and phosphate, in a similar strategy to the formation of glycoside bonds between two sugars catalyzed by glycosynthases [28]. Thus, three nucleophile mutants (Hsero1941-D255G, Hsero1941-D255A and Hsero1941-D255S) were constructed by site-directed mutagenesis and over-expressed in *E. coli* (Fig. S5). The mutants were inactive against *p*NP-β-D-GlcNAc*p*, which was consistent with data previously reported for nucleophile mutants of β-*N*-acetylglucosaminidases from GH3 [15]. The reactions for the production of βGlcNAc 1*P* (**3**) from αFGlcNAc (**7**) were performed in 500 mM PBS and analyzed by ^{1}H NMR spectroscopy (Fig. 4). Two major signals were observed in the spectrum at 5.62 ($J_{1,2}$ = 2.7 and $J_{1,F}$ = 52.8 Hz) and 5.19 ($J_{1,2}$ = 3.4 Hz, H1$_\alpha$) ppm and were attributed to the anomeric protons of substrate αFGlcNAc (**7**) and its hydrolytic byproduct GlcNAc (**2**), respectively. However, a small signal was also noted at 4.93 (J = 8.3 Hz) ppm, similar to the chemical shift reported in the literature for the anomeric proton of βGlcNAc 1*P* (**3**) [16]. The correlation found at 4.93/1.85 ppm in the ^{1}H-^{31}P HMBC spectrum confirmed the formation of **3** in the reaction. Surprisingly, the anomeric signal corresponding to the glycosyl phosphate was also observed in the control reaction, indicating that the formation of this glycoside was not catalyzed by the mutants, but the intrinsic reactivity between **7** and phosphate under the reaction conditions. WaterLOGSY NMR experiments were performed in order to investigate whether αFGlcNAc (**7**) could bind the wild-type glycosidase and its mutants (Fig. S6). Although the mutants were not able to catalyze the synthesis of βGlcNAc 1*P* (**3**), the spectra indicated that the glycosyl fluoride bound the mutants and wild-type enzyme by the change of phase of signals at 5.62, 3.57 and 2.03 ppm, corresponding to H1, H4 and the *N*-acetyl of **7**, respectively. When the reaction between **7** and phosphate was performed in the presence of Hsero1941 (Fig. 4), the production of βGlcNAc 1*P* (**3**) was not detected, which was attributed to the hydrolysis of this product by the wild type β-*N*-acetylglucosaminidase.

Intrigued by the non-enzyme catalyzed nucleophilic substitution between αFGlcNAc **7** and phosphate, we performed a reaction in 50 mM Hepes buffer containing 2 M azide, a well-known good nucleophile (Fig. 7S). The βN$_3$GlcNAc **8** was produced as major product after 48 h, as identified by ^{1}H NMR analysis [29]. When the reaction was carried out in the presence of Hsero1941, GlcNAc **2** was the only product detected, indicating that **8** was hydrolyzed by the wild type enzyme after its formation in the reaction mixture. The hydrolytic activity of glycosidases against azido sugars has been reported in the literature [30].

3. Discussion

Hsero1941 is a glycosidase with higher specificity for glycoside substrates of GlcNAc than GalNAc. This result is consistent with β-*N*-acetylglucosaminidases from GH3, which have a preference for amino sugars with Glc instead of Gal configuration [17,18]. Hsero1941 showed activity against Glc, however, according to the k_{cat}/K_m values the preference for glycosides of Glc was less than that observed for the GH3 phosphorylase from *C. fimi* [16,19]. Although the anion rescue results have showed that phosphate was able to react with the glycosyl-enzyme intermediate, the revival of catalytic activity was similar to other anions tested. In addition, the presence of phosphate in the reaction had no significant effect on the enzyme activity. Those results, together with the identification of the reaction product by ^{1}H NMR, clearly demonstrated that Hsero1941 is a glycoside hydrolase with a retaining mechanism.

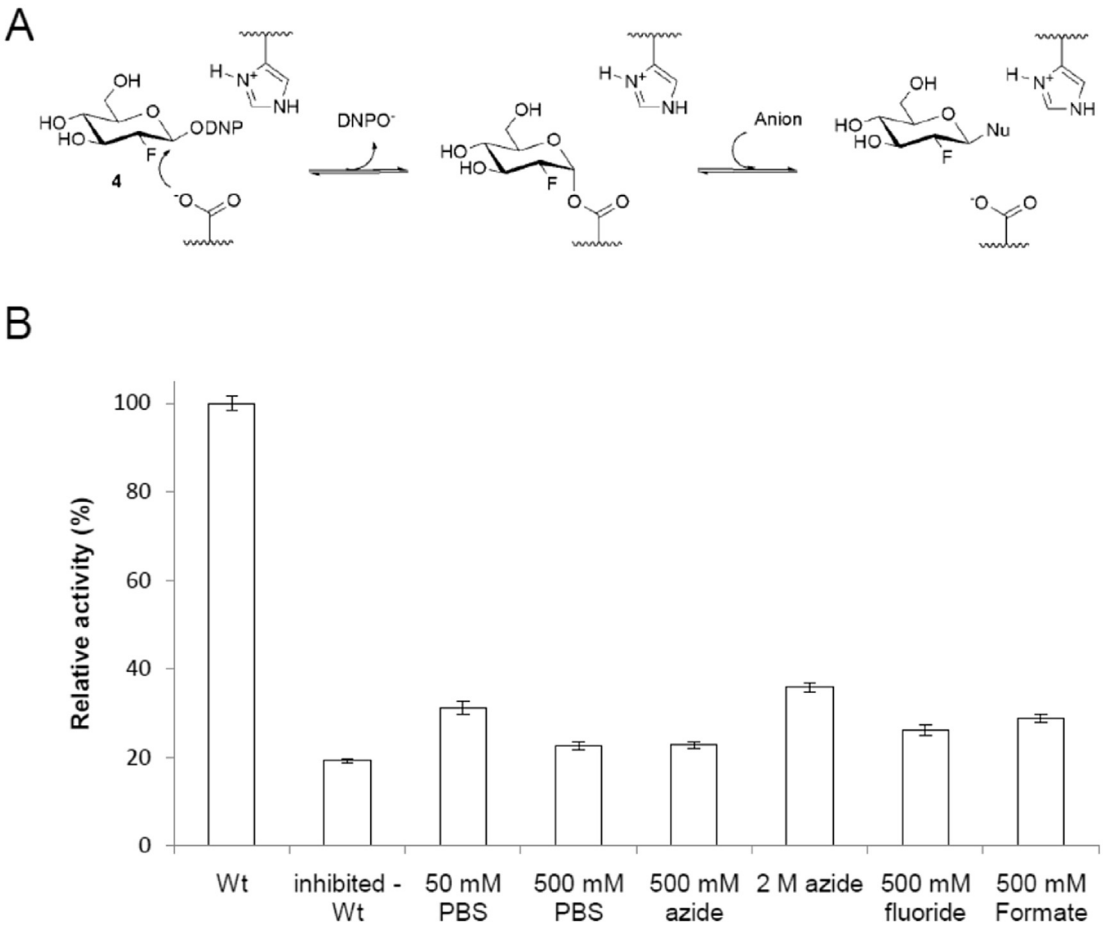


Fig. 2. Anion reactivation of covalently inhibited 2FGlc-Hsero1941. (**A**) Scheme showing the enzyme inhibition using DNP2FGlc (**4**) as a covalent inhibitor. (**B**) Relative activity of inhibited 2FGlc-Hsero1941 against *p*NP-β-D-GlcNA*cp* after incubation with several anions.

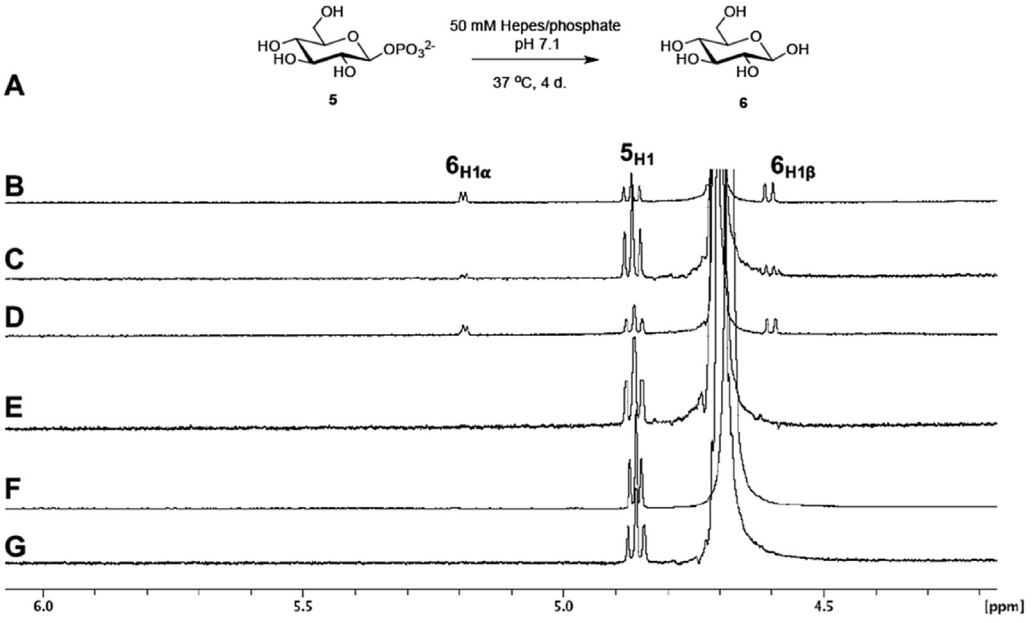


Fig. 3. ^{1}H NMR spectra showing hydrolysis of β-D-Glc 1*P* **5**. (**A**) Schematic hydrolysis of **5**. (**B**) 10 mM **5**, Hsero1941 70 μM. (**C**) 5 mM **5**, Hsero1941 10 μM. (**D**) 10 mM **5**, EryBI 70 μM. (**E**) 5 mM **5**, EryBI 10 μM. (**F**) 5 mM **5**, DesR 15 μM. (**G**) 5 mM **5**, without enzyme.

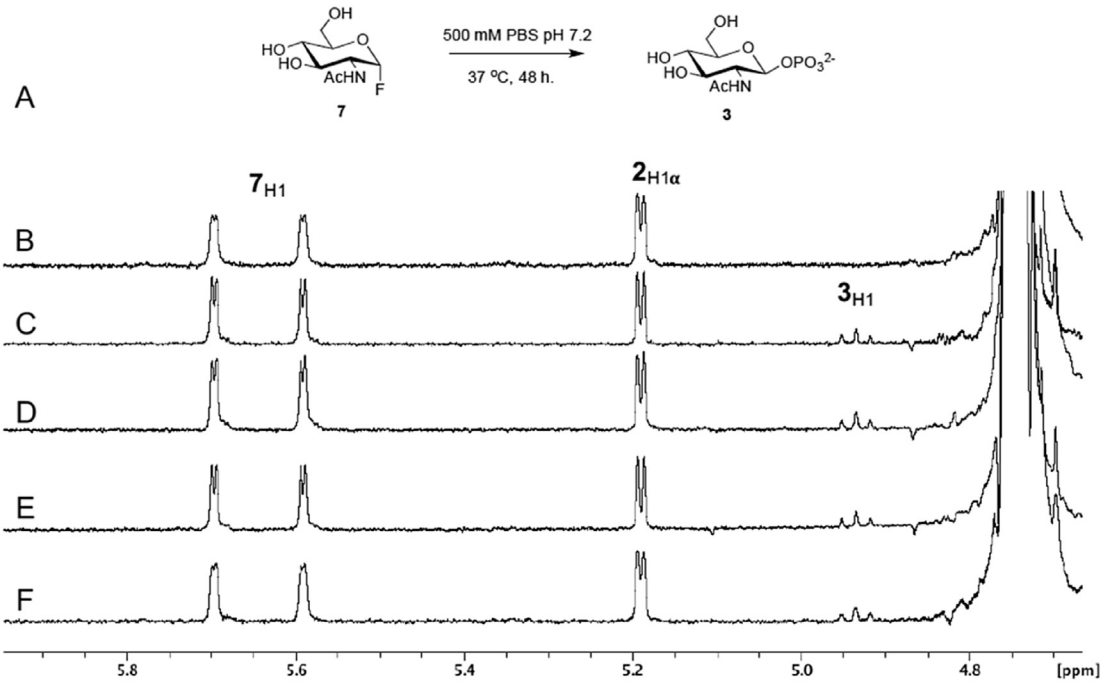


Fig. 4. Chemical synthesis and enzymatic hydrolysis of βGlcNAc 1*P* **3**. (**A**) Synthesis of **3** from αFGlcNAc **7**. ^{1}H NMR spectra showing products in reactions performed with 15 mM **7** and 30 μM of enzyme: (**B**) Hsero1941; (**C**) Hsero1941 D255S; (**D**) Hsero1941 D255A; (**E**) Hsero1941 D255G; (**F**) control reaction without enzyme.

It is worth noting that at lower enzyme concentrations, Hsero1941 was able to hydrolyze the charged substrate βGlc 1*P* **5**, while known GH3 β-glucosidases DesR and EryBI were inactive against this substrate. Although the hydrolytic activity for EryBI was observed at higher concentrations, the substrate consumption for either enzyme was not complete after 4 days of incubation. It has been proposed that some glycosidases containing His or Tyr as catalytic residues instead of the usually found Glu or Asp, could accept charged substrates due to a smaller Coulombic repulsion within the catalytic site [16,31]. Indeed, DesR and EryBI show Glu/Asp and Asp/Asp dyads, respectively, in the catalytic site [26,27], while Hsero1941 presents a His and Asp, which might explain the affinity difference for βGlc 1*P* (**5**). However, the identity of catalytic residues could not be the only structural requirement to rationalize the catalysis of charged phospho-substrates by glycosidases. For instance, an α-retaining sucrose phosphorylase from GH13 has Glu and Asp as catalytic residues that do not hamper the nucleophilic phosphate attack on the glycosyl-enzyme intermediate [32]. Furthermore, the first mannosidases from GH130 [33], a family known to contain inverting mannoside phosphorylases have been characterized. These glycoside hydrolases did not show conserved basic amino acid residues responsible for binding phosphate, and for this reason, a water molecule acts as nucleophile. Therefore, additional features in the tertiary structure of GH3 β-*N*-acetylglucosaminidases may also be responsible for the interaction of phospho-compounds and phosphate in the catalytic site. Further 3D studies could be useful to explain the presence of hydrolases and phosphorylases in this family.

The nucleophile mutants were not able to synthesize βGlcNAc 1*P* (**3**) employing αFGlcNAc (**7**) as donor. We did not observe glycosynthase activity using a broad library of glycosides and alcohols as acceptors (data not shown). The mutation of a glycosidase into a glycosynthase has been reported as an interesting approach in glycobiology to produce enzymes able to synthesize a variety of glycosidic structures [34]. However, not all glycosidases can produce active glycosynthases [26], indeed, EryBI remains the only active glycosynthase from GH3 [27].

The capacity of Hsero1941 to hydrolyze azido-, phospho- and *p*NP- glycosides indicated a broad aglycone site specificity, which may be associated with the recognition *in vivo* of different substrates containing GlcNAc. This result is consistent with β-*N*-acetylglucosaminidases from GH3, which are involved in bacterial cell wall recycling [9]. In addition, a recent study has been reported indicating that NagZ from *Neisseria gonorrhoeae* is involved in biofilm metabolism, suggesting a biological function for the enzymes from this family beyond that of peptidoglycan recycling [35]. However, it is not clear whether the ability of Hsero1941 to cleave the phosphorylated sugar substrates, especially βGlcNAc 1*P* is important in bacterial metabolism, since the βGlcNAc 1*P* motif is rare in the nature, being found only in some bacterial glycolipids [36]. Another possibility to consider is that glycosyl phosphate hydrolysis remains a promiscuous activity detected *in vitro*, which could be associated with the capacity to recognize phosphate groups by members of this family. The capacity of GH3 glucosidase EryBI to hydrolyze βGlc 1*P* indicates that the presence of a His acid/base catalyst is not a prerequisite for the recognition of charged substrates within the GH3 family.

In conclusion, Hsero1941 is a retaining beta-*N*-acetylglucosaminidase from GH3. The enzyme showed a broad aglycone site specificity, being able to recognize aryl glycosides and glycosyl phosphates as substrates for hydrolysis. These data indicate that all GH3 family members have hydrolytic activity, whilst only some of the family members have phosphorolytic activity, and that the presence of a histidine general acid/base is not predictive nor correlative with the observed phosphorolytic activity. Further studies are needed to determine whether the hydrolytic activity against glycosyl phosphates imparts physiological importance in bacterial metabolism.

4. Experimental

4.1. Cloning and mutagenesis of Hsero1941

The gene encoding the glycosidase was amplified from genomic DNA of *H. seropedicae* SmR1 using high-fidelity polymerase and

primers (Supplemental Table S2) containing *Nde*I and *Hin*dIII restriction sites. The gene was digested and ligated into a pET24b vector to express the protein with a *C*-terminal His_6-tag. Site-directed mutagenesis was performed using QuickChange Lightning kit (Agilent Technologies), according to the manufacturer's instructions. Primers were designed in order to introduce a silent mutation together with the exchange of target amino acid (Supplemental Table S2). The silent mutation added a new restriction site to assist in the identification of cells harboring the mutated vector.

4.2. Expression of Hsero1941 and its mutants

The recombinant enzymes were expressed in *E. coli* BL21 (DE3) cells according to previously reported procedures [26]. Briefly, the cells were grown (37 °C, 250 rpm) in Luria-Bertani media containing kanamycin (50 μM/mL) until an O.D (600 nm) of 0.7–0.9 was reached. Afterwards, IPTG was added to a final concentration of 1 mM, and the cultures incubated (24 h, 17 °C, 250 rpm) and subsequently centrifuged (4000*g*). The cell pellet was resuspended with lysis buffer, stirred (30 min, 0 °C), sonicated (5 × 5 s) and then centrifuged (13,000 rpm, 4 °C). The supernatant was loaded onto a HisTrap HP column (5 mL) and proteins were eluted using a stepwise imidazole gradient (25–250 mM). The collected tubes were analyzed by SDS-PAGE, pooled, concentrated using centrifugal filter devices (cut-off 10,000 Da) and desalted on a Sephadex G-25 column (10 mL) using 50 mM Hepes buffer pH 7.1 as eluent. The protein concentrations were calculated measuring the solution absorbance at 280 nm (ε_{280} = 29,700 $M^{-1}cm^{-1}$).

4.3. Steady state kinetic analysis

Continuous kinetic assays were performed in 96-well plates using a Molecular Devices Spectromax 384 UV spectrophotometer, monitoring the release of *p*NP at 400 nm (ε_{400} = 7280 $M^{-1}cm^{-1}$). Initial velocities were calculated in Softmax Pro V4.6. Assays were performed in 50 mM Hepes buffer, pH 7.1 containing 0.3 μM Hsero1941 and commercial *p*NP-β-D-GlcNAc*p* or *p*NP-β-D-Glc*p* as substrates ranging from 0.15 to 4.8 mM and 10–100 mM, respectively. For the reactions performed in the presence of phosphate, Hepes buffer was supplemented with PBS pH 7.2 (25, 50, 100 and 200 mM). Kinetic parameters were obtained from at least 2 different assays (n = 2 for each point) by fitting initial velocities data to the Michaelis-Menten equation using Grafit 5.0 (Erithacus software). Results were expressed as mean ± standard error of the mean (SEM).

4.4. Enzymatic activity in PBS buffer

To a solution containing *p*NP-β-D-GlcNAc*p* and PBS, the Hsero1941 was added. The resulting mixture was incubated at 37 °C, for 2 h, centrifuged using Nanosep 10 K Ω filter devices (cut-off 10,000 Da), lyophilized and analyzed by ^{1}H NMR spectroscopy.

4.5. Reactivation with anions

Hsero1941 (67 μM) was incubated with DNP2FGlc (see Supporting Information for the synthesis) (50 mM) in 50 mM Hepes buffer pH 7.1 (200 μL final volume) at 37 °C. The enzyme activity was monitored measuring the initial velocity as described above, employing *p*NP-β-D-GlcNAc*p* as substrate and using wild type enzyme, incubated without inhibitor, as a control. After 16 h, the inhibitor was removed by centrifugation using Nanosep 3 K Ω filter devices (cut-off 3000 Da) and washing with 50 mM Hepes buffer (4 × 400 μL). The enzyme concentration was estimated measuring the activity and comparing with the activity level before the centrifugation step. Afterwards, inhibited enzyme aliquots (final concentration = 1.37 μM) were added into wells containing 50 mM Hepes buffer pH 7.1 and different anions added (azide, phosphate, fluoride and formate, final volume = 100 μL). The reactions (n = 6) were incubated 19 h at room temperature, and enzyme activity was estimated measuring initial velocity after addition of 100 μL *p*NP-β-D-GlcNAc*p* (3.6 mM). Results were expressed as mean relative to the activity of non-inhibited wild type enzyme.

4.6. Hydrolysis of βGlc 1P

To a solution containing βGlc 1*P* in 50 mM Hepes/phosphate pH 7.1, the enzyme was added. The resulting mixture was incubated at 37 °C, for 4 days, lyophilized and analyzed by ^{1}H NMR spectroscopy.

4.7. Chemical synthesis and enzymatic hydrolysis of βGlcNAc 1P and βN_3GlcNAc

Wild type and mutants (150 μM) in 50 mM Hepes pH 7.1, 160 mM αFGlcNAc (see supplemental data for the synthesis) in water, 2 M PBS pH 7.2 and 4 M NaN_3 in 50 mM Hepes pH 7.1 stock solutions were prepared. To a solution containing 15 mM αFGlcNAc in 500 mM PBS or 2 M NaN_3, enzymes (30 μM) were added. The resulting mixture was incubated at 37 °C, for 2 days, lyophilized, and analyzed by ^{1}H NMR spectroscopy.

4.8. NMR analysis

NMR spectra were acquired on a Bruker AV 500 or 300 spectrometer, operating at 500 or 300 MHz for ^{1}H. The chemical shifts were measured relative to internal acetone (δ = 2.208 ppm for ^{1}H). The data were analyzed using Bruker Topspin 3.5 software.

Acknowledgment

We thank the Natural Sciences and Engineering Research Council of Canada and CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brasil (scholarship 205131/2014-0) for funding. The authors are thankful to Prof. Marcelo M. dos Santos for the cultivation of *H. seropedicae* SmR1 and extraction of the genomic DNA.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carres.2016.09.015.

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