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The identification and molecular characterization of the first archaeal bifunctional exo-β-glucosidase/N-acetyl-β-glucosaminidase demonstrate that family GH116 is made of three functionally distinct subfamilies

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ABSTRACT

Background: β-N-acetylhexosaminidases, which are involved in a variety of biological processes including energy metabolism, cell proliferation, signal transduction and in pathogen-related inflammation and autoimmune diseases, are widely distributed in Bacteria and Eukaryotes, but only few examples have been found in Archaea so far. However, N-acetylgluco- and galactosamine are commonly found in the extracellular storage polymers and in the glycans decorating abundantly expressed glycoproteins from different Crenarchaeota *Sulfolobus* sp., suggesting that β-N-acetylglucosaminidase activities could be involved in the modification/recycling of these cellular components.
Methods: A thermophilic β-N-acetylglucosaminidase was purified from cellular extracts of *S. solfataricus*, strain P2, identified by mass spectrometry, and cloned and expressed in *E. coli*. Glycosidase assays on different strains of *S. solfataricus*, steady state kinetic constants, substrate specificity analysis, and the sensitivity to two inhibitors of the recombinant enzyme were also reported.
Results: A new β-N-acetylglucosaminidase from *S. solfataricus* was unequivocally identified as the product of gene *sso3039*. The detailed enzymatic characterization demonstrates that this enzyme is a bifunctional β-glucosidase/β-N-acetylglucosaminidase belonging to family GH116 of the carbohydrate active enzyme (CAZy) classification.
Conclusions: This study allowed us to propose that family GH116 is composed of three subfamilies, which show distinct substrate specificities and inhibitor sensitivities.
General significance: The characterization of SSO3039 allows, for the first time in Archaea, the identification of an enzyme involved in the metabolism β-N-acetylhexosaminide, an essential component of glycoproteins in this domain of life, and substantially increases our knowledge on the functional role and phylogenetic relationships amongst the GH116 CAZy family members.

1. Introduction

Glycoside hydrolases (GHs), the enzymes that hydrolyze glycoconjugates, oligo- and polysaccharides, are widespread in all living domains and are involved in hundreds of enzymatic reactions essential for the cell. These enzymes have been segregated in sequence-based families, which have been shown to group together enzymes of differing substrate specificities [1]. Subsequent phylogenetic analyses have shown that multifunctional families group distinct subfamilies [2,3]. GHs constitute the most numerous category of carbohydrate active enzymes (CAZymes) listed in the carbohydrate active enzyme database (CAZy, http://www.cazy.org/) [4]. The number of GHs that have been biochemically characterized is very small compared to the number of available GH

Abbreviations: CAZy, carbohydrate active enzyme classification; X-GlcNAc, 5-bromo-4-chloro-indolyl-N-acetyl-β-D-glucosaminide; 4NP-GlcNAc, 4-nitropheny-N-acetyl-β-D-glucosaminide; MS, mass spectrometry; 4Np-Glc, 4Np-β-glucopyranoside; Glc, glucose; GlcNAc, N-acetylglucosamine; MU-Glc, 4-Methylumbelliferyl-β-D-glucoside; MU-GlcNAc, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide; Glc4, cellotetraose; Glc5, cellopentaose; GlcNAc4, tetra-*N*-acetylchitotetraose; GlcNAc5, penta-*N*-acetylchitopentaose; 2Np-Cel, 2Np-β-cellobioside; 4Np-Chit, 4Np-*N*,*N*′-diacetyl-β-chitobioside; o.n., 'overnight; HPAEC-PAD, High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection; NB-DNJ, N-butyldeoxynojirimycin; CBE, conduritol β-epoxide; FCE, free cell extract; HIC, Hydrophobic Interaction Chromatography

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sequences and this gap is continuously widening because access to (meta)genomic sequences is considerably faster and cheaper than time-consuming structure/function studies. Therefore, the characterization of novel CAZymes and their assignment to families and subfamilies are very useful to better understand the structure/function characteristics of the enzymes belonging to an existing family, to facilitate the discovery of enzymes with similar properties and to improve functional predictions during genome annotations. In these regard, the biochemical characterization of CAZymes from extremophilic Archaea is often the only instrument to shed light on the function and physiological relevance of these enzymes, since molecular genetic tools are still restricted to few species and are hampered by the harsh growth conditions [5].

Whilst CAZymes are found in all domains of life, the distribution of GHs across Archaea is unequal with several archaeal genomes completely devoid of known GHs [6] and, when the enzymes are present, they have peculiar family distribution. Thus, few examples of archaeal enzymes capable of hydrolysing β-N-acetylhexosaminides have been described so far. In the CAZy database these activities include β-N-acetylhexosaminidases (EC 3.2.1.52, in families GH3, GH20, and GH84 [7]), endo-N-acetyl-β-glucosaminidases or chitinases (EC 3.2.1.14, in families GH18 and GH48), mannosyl-glycoprotein endo-β-N-acetylglucosaminidases (EC 3.2.1.96, in families GH18 and GH85), β-1,6-N-acetylglucosaminidases or lacto-N-biosidases (EC 3.2.1.140, in family GH20), hyaluronoglucosaminidase (EC 3.2.1.35, in family GH84), and other enzymes, lacking a specific EC number, including β-6-SO_3-N-acetylglucosaminidases (GH20) and peptidoglycan hydrolase with endo-β-N-acetylglucosaminidase specificity (GH73). The only known archaeal enzymes able to cleave β-N-acetylhexosaminides are the chitinases from the Euryarchaea *Thermococcus chitinophagus*, *T. kodakaraensis* KOD1, *Pyrococcus furiosus*, and *Halobacterium* sp. strain NRC-1 [8–12] and from the Crenarchaeon *Sulfolobus tokodaii* [13]. Chitin is an important structural component of cell walls in fungi and yeast and in the exoskeletons of insects and crustacean, therefore, the presence of the GH18 chitinolytic enzymes in euryarchaeal marine organisms, is not surprising. Instead, the *in-vivo* function of the distantly related chitinase from *S. tokodaii*, which is not assigned to any CAZy family, is more difficult to predict [13], as this organism lives in sulphur-rich hot acid springs, where it has few opportunities of using chitin as carbon source than other soil microorganisms [14].

In Archaea, N-acetylgluco- and galactosamine (GlcNAc and GalNAc, respectively) have been identified in exopolysaccharides and biofilms [15,16], in glycans N-linked to proteins [17–20], and the biosynthetic pathway of GlcNAc has been characterized [21,22]. More specifically, within the crenarchaeal group of *Sulfolobales*, GlcNAc has been proved to be a component of the extracellular polymeric substance (EPS) composing their biofilms [23,24] and of the N-glycan decorating the cytochrome $b_{558/566}$ and the Surface (S)-layer protein from *S. acidocaldarius*, and an ABC transporter from *S. solfataricus* [25–27]. It has been pointed out that the EPS from *Thermococcus* and *Sulfolobus* genera can also act as extracellular storage polymers, therefore being substrates for cognate GHs [28]. In addition, since glycosylated S-layer proteins and ABC transporters are abundantly expressed in *Sulfolobus* sp., [26,27,29], the GHs from this organism could be involved in the recycling of the cell components similarly to Bacteria [30]. This has been proposed for the thermoacidophilic archaeon *S. solfataricus*, strain P2, which has 27 potential glycoside hydrolases from 14 different GH families (CAZy classification, June 2013), of which a β-glycosidase from GH1 [31] and an α-mannosidase from GH38 [32], could be involved *in-vivo* in the modulation of the sugar composition of the EPS and in the de-mannosylation of the glycan tree of the extracellular glycoproteins [33]. So far, no β-N-acetylglucosaminidase activity has been identified in this microorganism, thus, in the framework of our studies on the identification of novel GHs from extremophiles [31,32,34–37], we embarked in testing if such an important enzymatic activity was expressed in free cell extracts of *S. solfataricus*, strain P2.

We report here the purification of the native enzyme, the identification by LC/MS/MS of its amino acid sequence, the cloning and expression of its gene, and the detailed enzymatic characterization. We found that the β-N-acetylglucosaminidase activity was associated with the product of gene *sso3039* and the enzyme belongs to a recently described family (GH116), which we defined based on the characterization of a bifunctional β-glucosidase/β-xylosidase also from *S. solfataricus* strain P2 [38]. The present study allowed us to propose that family GH116 is composed of three subfamilies, which show distinct substrate specificities and inhibitor sensitivities.

2. Materials and methods

2.1. Materials

All commercially available substrates were purchased from Sigma-Aldrich and Carbosynth. The synthetic oligonucleotides were from PRIMM (Italy).

2.2. Strains, media and standard growth conditions

S. solfataricus strains P2, 98/2, and PBL2025 were grown at 80 °C, pH 3.5 in Brock's salt medium supplemented with yeast extract, sucrose, and casamino acids (0.1% each) [39]. The growth of cells was monitored spectrophotometrically at 600 nm and the cells were harvested at the early stationary phase (0.7–1.0 OD) by centrifugation at 5000 ×*g* for 15 min at 4 °C.

2.3. Isolation of β-N-acetylglucosaminidase from S. solfataricus P2

The isolation of β-N-acetylglucosaminidase activity from *S. solfataricus* P2 cellular extracts was performed by following the enzymatic activity on the chromogenic substrates 4Np-GlcNAc and X-GlcNAc at 60 °C. A culture of 5.0 L of *S. solfataricus* P2 was centrifuged and the cellular pellet was resuspended in 2 mL g^{-1} cells of PBS buffer (20 mM sodium phosphate buffer, pH 7.3, 150 mM NaCl) supplemented with 0.1% Triton X-100. The cells were lysated with three cycles of freeze–thawing (5 min at −70 °C; 5 min at 37 °C) and centrifuged at 10,000 ×*g* for 30 min at 4 °C. The free cell extract (FCE) was loaded on a High Load 16/10 Q-Sepharose High Performance column (GE-Healthcare) equilibrated in 20 mM phosphate buffer, pH 7.5 (Buffer A) at a flow rate of 3 mL min^{-1}. The run was performed with an initial step of extensive wash with Buffer A (3-column volumes) followed by a linear ionic strength gradient from 0 to 1 M NaCl in Buffer A (3-column volumes) and a final step with 1 M NaCl in Buffer A (2-column volumes). At these conditions, the β-N-acetylglucosaminidase activity was found primarily in the fractions eluted at about 150 mM NaCl. Active fractions were pooled, equilibrated in 1 M ammonium sulphate and loaded on a HiLoad 26/10 Phenyl Sepharose High performance (GE-Healthcare), equilibrated at a flow rate of 3 mL min^{-1} with Buffer 'A supplemented with 1 M ammonium sulphate. After 1-column volume of loading buffer, the protein was eluted with a two-step gradient of water (0–80%, 2-column volumes; 80–100%, 3-column volumes) 'followed by a final step at 100% of water (2-column volumes); the protein eluted in about 85% water. Active fractions were pooled, dialyzed against PBS buffer, and concentrated by ultrafiltration on an Amicon YM30 membrane (cut off 30,000 Da). After concentration, the sample 'was loaded on a Superdex 200 HR 10/300 gel filtration column (GE-Healthcare) and the run was performed at a flow rate of 0.5 mL min^{-1} in PBS buffer. Active fractions were pooled and concentrated. The protein concentration was determined with the Bradford assay [40].

2.4. Gel staining of β-N-acetylglucosaminidase activity

The activity on 5-bromo-4-chloro-indolyl-N-acetyl-β-D-glucosaminide (X-GlcNAc) was analysed on 10% SDS-PAGE gel by loading the non heat-denatured samples. After the run, the gel was washed with water, equilibrated in 50 mM phosphate pH 6.5 and then incubated 16 h at 65 °C in 50 mM phosphate pH 6.5 with 2 mg mL^{-1} of X-GlcNAc and 10% methanol.

2.5. Protein identification by mass spectrometry

TPCK-treated trypsin and dithiothreitol (DTT) were from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were of the highest purity available from Carlo Erba (Milan, Italy). Protein samples were reduced by incubation in 10 mM DTT for 2 h min at 37 °C. The cysteines were alkylated by incubation in 5 mM iodoacetamide for 30 min at room temperature in the dark. Enzymatic digestion was carried out with trypsin (1/50; w/w) in 50 mM ammonium bicarbonate pH 8.5 at 37 °C for 16 h.

2.6. Nano LC mass spectrometry

A mixture of peptide solution was analysed by LC/MS/MS analysis using a 3520 chipQ-TOF (Agilent) equipped with an 1100 nano HPLC system (Agilent Technologies). The mixture was loaded on an Agilent reverse-phase pre-column cartridge (Zorbax 300 SB-C18, 5 × 0.3 mm, 5 μm) at 10 μL min^{-1} (A solvent 0.1% formic acid, loading time 5 min). Peptides were separated on an Agilent reverse-phase column (Zorbax 300 SB-C18, 150 mm × 75 μm, 3.5 μm), at a flow rate of 0.3 μL min^{-1} with a 0% to 65% linear gradient in 60 min (A solvent 0.1% formic acid, 2% ACN in MQ water; B solvent 0.1% formic acid, 2% MQ water in ACN). Nanospray source was used at 2.5 kV with liquid coupling, with a declustering potential of 20 V, using an uncoated silica tip from New Objectives (O.D. 150 μm, I.D. 20 μm, T.D. 10 μm). Data were acquired in information-dependent acquisition (IDA) mode, in which a full scan mass spectrum was followed by MS/MS of the 5 most abundant ions (2 s each). In particular, spectra acquisition of MS–MS analysis was based on a survey Enhanced MS Scan (EMS) from 400 m/z to 1400 m/z at 4000 amu/s. This scan mode was followed by an Enhanced Resolution experiment (ER) for the five most intense ions and then MS^2 spectra (EPI) were acquired using the best collision energy calculated on the bases of m/z values and charge state (rolling collision energy) from 100 m/z to 1400 m/z at 4000 amu/s.

2.7. MASCOT analysis

Spectral data were analysed using Mass Hunter (version B.02.00); MS–MS centroid peaks were threshold at 0.1% of the base peak. MS/MS spectra having less than 10 peaks were rejected. MS/MS spectra were searched against 3015 sequences contained in a *S. solfataricus* database, using the licenced version of Mascot 2.4.0 version (Matrix Science). MASCOT search parameters were: allowed number of missed cleavages 3; enzyme trypsin; fixed modification, carbamidomethylation; variable post-translational modifications, methionine oxidation, pyro-glu N-term Q; peptide tolerance 10 ppm and MS/MS tolerance 0.6 Da; peptide charge, from +2 to +3. Spectra with a MASCOT score <20 having low quality were rejected. The score used to evaluate the quality of matches for MS–MS data was higher than 30.

2.8. Cloning and expression in E. coli

The *sso3039* gene was amplified by PCR from the genome of *S. solfataricus* P2 using the synthetic oligonucleotides 3039TOPO5′ (5′-CACCATGAAATACAAATACTCTTAT-3′) and 3039TOPO3′His (5′-AAATTCAATCTCTTCATTTTCCC-3′). The amplification reaction was performed with the PfuUltra II Fusion HS DNA Polymerase (Stratagene) by using the following programme: hot start 5 min at 95 °C; 5 cycles 1 min at 95 °C, 1 min at 50 °C and 3 min at 72 °C; 30 cycles 1 min at 95 °C, 1 min at 55 °C, and 3 min at 72 °C; final extension 10 min at 72 °C. The DNA fragment obtained was cloned in the expression vector pET101/D-TOPO (Invitrogen), obtaining the recombinant plasmid pET101/D-TOPO-SSO3039, in which the SSO3039 ORF is under the control of the isopropyl-1-thio-β-D-galactopyranoside (IPTG) inducible T7 RNA polymerase promoter and the C-terminal of the protein was fused to V5 epitope and 6xHis tag. PCR-generated construct was verified by sequencing and the ORF was expressed in *E. coli* cells, strain BL21 star (DE3) (Invitrogen), according to the manufacturer.

2.9. Protein purification

E. coli BL21 star (DE3) cells transformed with pET101/D-TOPO-SSO3039 were grown at 37 °C in 4.5 L of Luria–Bertani (LB) broth supplemented with ampicillin (50 μg mL^{-1}). Growth was allowed to proceed for 16 h without the addition of IPTG, and cells were harvested by centrifugation at 5000 ×*g*. The resulting cell pellet was resuspended in 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl (Buffer B) with a ratio of 5 mL g^{-1} cells and then was incubated at 37 °C for 1 h with 20 mg of lysozyme (Fluka) and 25 U g^{-1} cell of Benzonase (Novagen). The cells were lysed by French cell pressure treatment and cell debris was removed by centrifugation at 10,000 ×*g* for 30 min. The FCE was loaded on a His Trap FF crude column (GE-Healthcare) equilibrated with Buffer B. After an initial wash-step (20-column volumes) with buffer B, the protein was eluted with a two-step gradient of imidazole in Buffer B (250 mM imidazole, 20-column volumes; 500 mM imidazole, 15-column volumes) at a flow rate of 1 mL min^{-1}. The protein was eluted at 250 mM imidazole. The active fractions were pooled, dialyzed against PBS buffer and then heat-fractionated for 20 min at 80 °C. The resulting supernatant was concentrated to 2.82 mg mL^{-1} and applied on a HiLoad 16/60 Superdex 200 gel filtration column (GE-Healthcare) equilibrated with PBS buffer at a flow rate of 1 mL min^{-1}. The active fractions were pooled, concentrated and stored at 4 °C. After this procedure rSSO3039 was more than 95% pure by SDS-PAGE.

2.10. Molecular mass determination

The molecular mass of native and recombinant SSO3039 was determined by gel filtration on a Superdex 200 HR 10/300 FPLC column (GE-Healthcare) performed as described above (Section 2.9). Molecular weight markers were apoferritin (443 kDa), amylase (200 kDa), albumin (66 kDa) and ribonuclease A (13.7 kDa).

2.11. Temperature and pH influence on rSSO3039 activity

The temperature profile of rSSO3039 activity was determined in the range of 40–70 °C using the standard assay conditions. Thermal stability was evaluated by incubating pure rSSO3039 (0.33 mg mL^{-1}) in PBS buffer, pH 7.3, at the indicated temperatures. At intervals, aliquots of 6 μL (2 μg enzyme) were withdrawn, transferred in ice and assayed at standard conditions as described below (Section 2.12). The residual activity was expressed as a percentage of the maximal enzymatic activity measured before the incubation at indicated temperatures; pH and buffered saline optima were determined by assaying rSSO3039 in 50 mM of the indicated buffers at different pHs in standard conditions.

2.12. Kinetic characterization of rSSO3039

The standard assay for rSSO3039 activity was performed in 50 mM citrate phosphate buffer, pH 4.0 on 4Np-GlcNAc at the final concentration of 12 mM. Typically, in each assay, we used about 2–5 μg of rSSO3039, in the final volume of 0.2 mL. For *S. solfataricus* free cell

Table 1
Purification of native and recombinant SSO3039.

	Volume (mL)	[Protein] (mg mL^{-1})	Total proteins (mg)	Activity[a] (U mL^{-1})	Total activity[a] (U)	Specific activity[b] (U mg^{-1})	Yields (%)	Purification folds
S. solfataricus[a]								
Free cell extract	4	53	212	0.30	1.20	5.5 10^{-3}	100	1
Anionic exchange	13	1.40	18.20	0.05	0.60	0.03	50	5.5
Hydrophobic chromatography	2.5	0.32	0.80	0.06	0.16	0.20	13	36
Gel filtration	0.5	0.04	0.02	0.05	0.03	1.30	2.50	236
E. coli[c]								
Free cell extract	116	20.60	2390	0.4	43	0.018	100	1
His trap	15	4.60	69	2.7	40	0.58	93	32
Heating step	14	1.10	15	2.5	34.5	2.3	80	128
Gel filtration	5	0.33	1.6	2.5	12.4	7.5	29	417

[a] Data from a 5 Lt culture.
[b] The enzyme purified from *S. solfataricus* and from *E. coli* were assayed in 50 mM sodium phosphate buffer, pH 6.5 and in 50 mM citrate/phosphate buffer, pH 4.0, respectively, at 60 °C for 2 min.
[c] Data from 4.5 Lt culture.

extracts (FCE) or FPLC fractions suitable amounts of the sample were incubated in 50 mM sodium phosphate buffer pH 6.5. After 2 min of incubation at 60 °C, the reaction was blocked in ice by adding 0.8 mL of 1 M sodium carbonate pH 10.2. The absorbance was measured at 420 nm at room temperature and the molar extinction coefficient was 17.2 $mM^{-1} \times cm^{-1}$. Assays on 4-Methylumbelliferyl-β-D-glucoside (MU-Glc) and 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (MU-GlcNAc) were performed in standard conditions in 0.25 mL by using 0.3 μg rSSO3039. The reaction was blocked by adding 0.5 mL of 0.1 M Glycine-NaOH. The 4-methylumbelliferone released was followed with a JASCO fluorometer with an excitation at 384 nm and an emission at 450 nm. Enzymatic assays on cellobiose and *N,N′*-diacetylchitobiose were performed in standard conditions in 0.1 mL containing 13 μg rSSO3039. The release of glucose and N-acetylglucosamine was monitored by the GOPOD kit (Megazyme) and by the Morgan–Elson method, respectively [41,42].

In all of the assays, spontaneous hydrolysis of the substrate was subtracted by using appropriate blank mixtures without the enzyme. One enzymatic unit is defined as the amount of enzyme catalysing the conversion of 1 μmol of substrate into product in 1 min, at the indicated

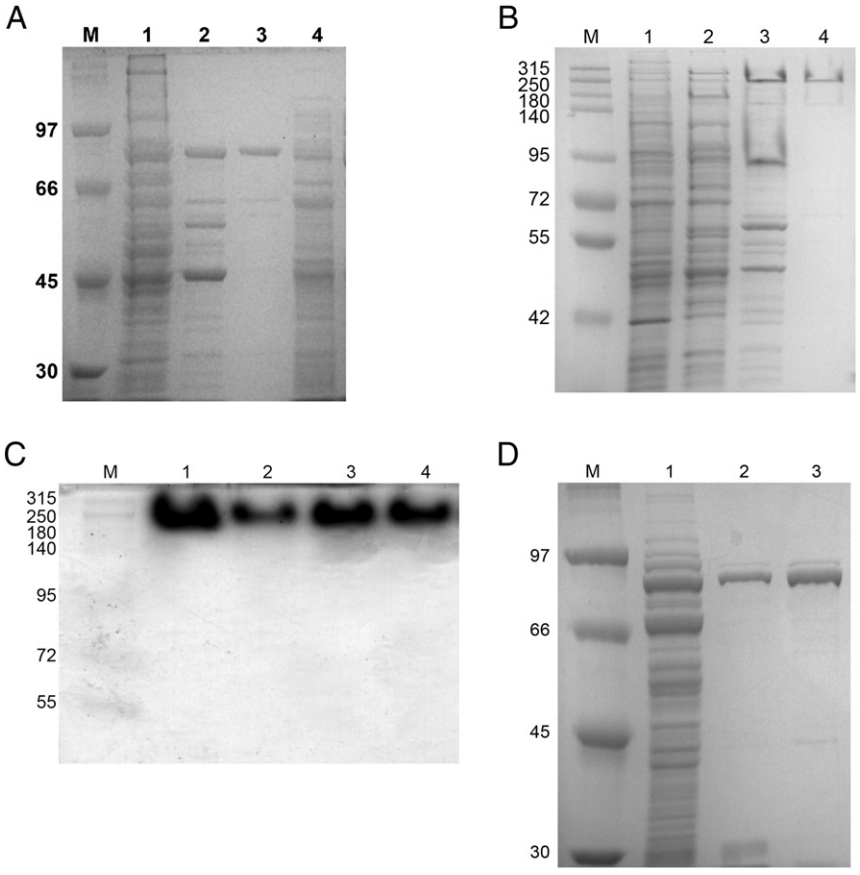

Fig. 1. SDS-PAGE of native and recombinant N-acetylglucosaminidase. (A): Coomassie staining of denaturated native samples. M: molecular weight markers; lane 1: anion exchange, lane 2: HIC; lane 3: gel filtration, lane 4: FCE of *S. solfataricus* P2. (B) Coomassie staining of non heat-denatured native samples. (C) Activity staining with X-β-GlcNAc of non heat-denatured native samples. M: molecular weight markers; lane 1: FCE of *S. solfataricus* P2, lane 2: anion exchange, lane 3: HIC; lane 4: gel filtration. (D) Coomassie staining of heat-denatured recombinant SSO3039 samples. M: molecular weight markers; lane 1: active pool from Ni-affinity chromatography; lane 2: thermal precipitation; lane 3: gel filtration.

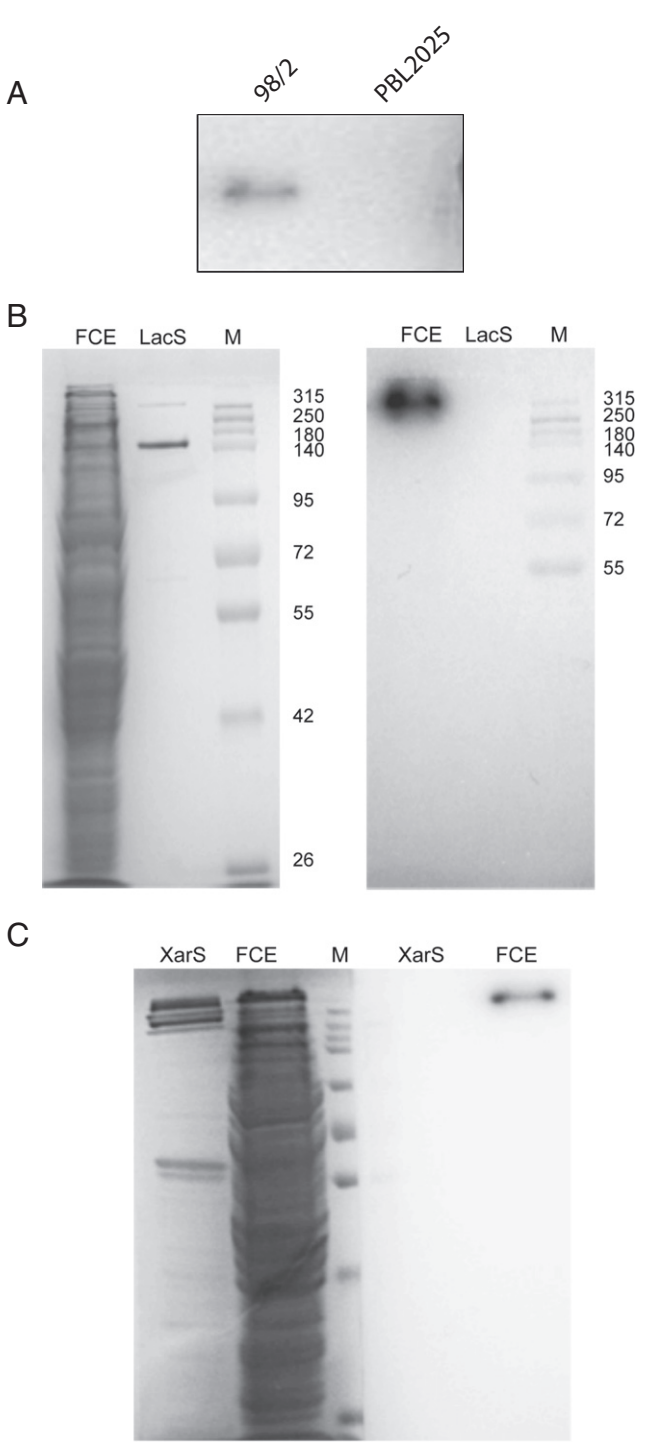

Fig. 2. SDS-PAGE of carbohydrate active enzymes from *S. solfataricus*. (A) Activity staining of FCE from *S. solfataricus* 98/2 (596 μg) and PBL2025 (434 μg). (B) Coomassie and activity staining (left and right panels, respectively) of 98/2 FCE (434 μg) and purified LacS (2 μg); (C) coomassie and activity staining (left and right panels, respectively) of 98/2 FCE (434 μg) and purified XarS (4.74 μg). Samples were not heat-denatured before loading.

conditions. All kinetic data were calculated as the average of at least two experiments and were plotted and refined with the programme GraphPad Prism.

The kinetic constants of rSSO3039 were measured at standard conditions by using concentrations of substrate ranging between 0.5 and 20 mM for 4Np-GlcNAc and 0.1 and 15 mM for 4Np-Glc, 0.05 and

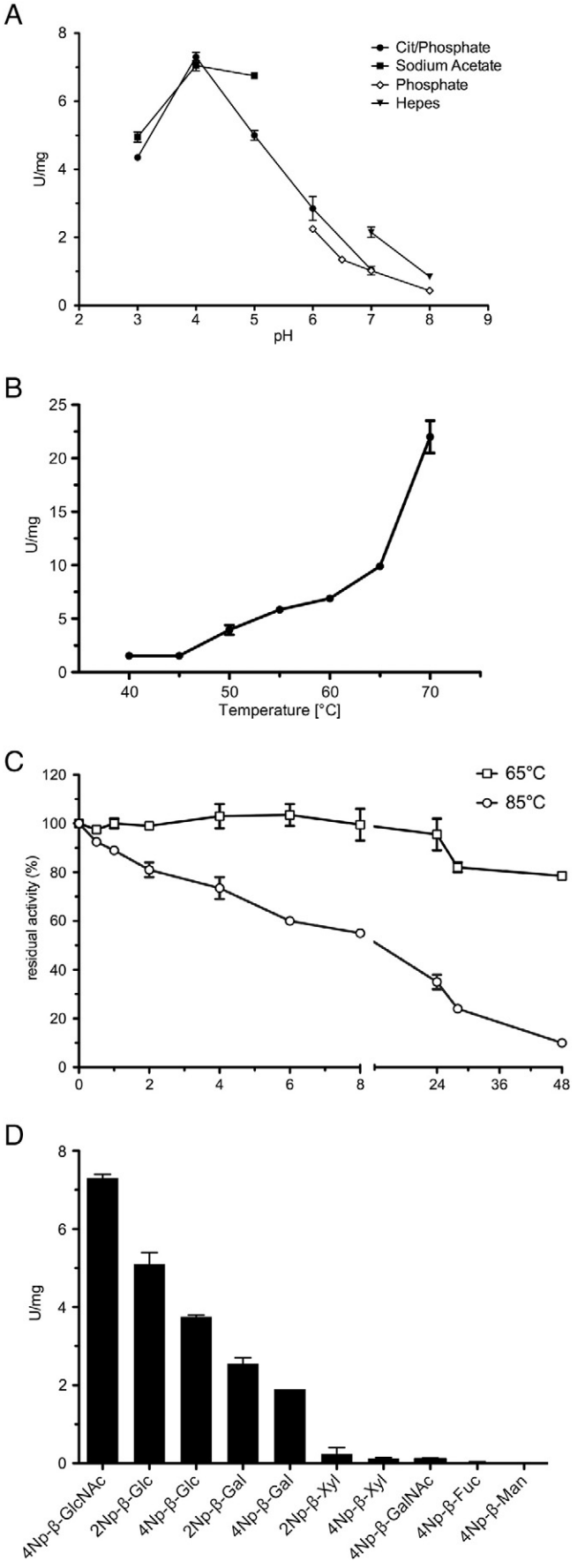

Fig. 3. Enzymatic characterization of SSO3039. (A) pH dependence. (B) Thermal activity. (C) Thermal stability at 65 °C and 85 °C (squares and circles, respectively). (D) Substrate specificity.

Table 2
Steady state kinetic constants of recombinant SSO3039.

Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
4Np-GlcNAc	19.2 ± 0.5	4.15 ± 0.3	1.6
4Np-Glc	9.15 ± 0.2	0.8 ± 0.1	11.4
MU-GlcNAc	5.4 ± 0.1	1.1 ± 0.1	4.9
MU-Glc	8.8 ± 0.5	0.9 ± 0.1	9.9
Cellobiose	1.8 ± 0.1	2.1 ± 0.2	0.9
N,N′-diacetylchitobiose	0.56 ± 0.03	10.8 ± 1.5	0.05

4 mM for MU-Glc, 0.25 and 15 mM for MU-GlcNAc, 1 and 40 mM for cellobiose and 2 and 30 mM for *N,N′*-diacetylchitobiose.

The substrate specificity of rSS3039 was determined by analysing the activity on different aryl-glycosides (final concentration 12 mM) in standard conditions.

The activity of rSSO3039 on tetra- and pentasaccharides of glucose and *N*-acetylglucosamine (Glc_4, Glc_5, $GlcNAc_4$ and $GlcNAc_5$) and on 2Np-β-cellobioside and 4Np-*N,N′*-diacetyl-β-chitobioside (4Np-Cel and 4Np-Chit, respectively) was measured by assaying the enzyme (5 μg) at standard conditions; aliquots were withdrawn at indicated times and stored at −20 °C. The analysis of the reaction products was performed by High Performance Anion Exchange chromatography with Pulsed Amperometric Detection (HPAEC-PAD) equipped with a PA200 column (Dionex, USA). HPAEC-PAD analyses of the reactions on 2Np-cel and gluco-oligosaccharides were performed at a flow rate of 0.5 mL min^{-1} in 100 mM NaOH/10 mM sodium acetate for 6 min followed by a two-step gradient of sodium acetate (10–100 mM in 10 min; 100–150 mM in 4 min); instead, an isocratic elution (10 mM NaOH for 40 min) was applied for the analysis of the products of the hydrolysis from *N*-acetyl-chitooligosaccharides and 4Np-Chit.

2.13. Inhibition of rSSO3039

To evaluate the inhibition of rSSO3039 by N-butyldeoxynojirimycin (NB-DNJ) and conduritol β-epoxide (CBE), the enzyme (0.08 mg mL^{-1}) was incubated in the presence of 0.1–50 μM NB-DNJ or 2–15 mM CBE in 50 mM phosphate buffer, pH 7.0, in a final volume of 60 μL. After incubation at 45 °C for 30 min, aliquots of 25 μL were withdrawn and the residual activity was measured in standard conditions. The residual activity was expressed as a percentage of the maximal activity measured in the same conditions in the absence of the inhibitor.

3. Results and discussion

3.1. Identification of N-acetylglucosaminidase activity in S. solfataricus extracts

Assays on free cell extracts of *S. solfataricus*, strain P2, on 12 mM 4-nitropheny-N-acetyl-β-D-glucosaminide (4NP-GlcNAc), revealing low (5.5×10^{-3} U mg^{-1}) but detectable activity, allowed us to set up a purification procedure summarized in Table 1. The steps used, including Q-sepharose anionic exchange, hydrophobic, and gel filtration chromatographies, increased by >200-fold the purification of the enzyme, which, however, was not pure to homogeneity (Fig. 1A). Nevertheless, the activity staining of the SDS-PAGE by using X-GlcNAc, revealed a single clear band of high molecular mass (Fig. 1B and C), confirming the results of the gel filtration in native conditions that showed a molecular mass of 271 ± 18 kDa (Supplementary Fig. S1A), and indicating that a single β-N-acetylglucosaminidase activity was present in *S. solfataricus* extracts.

To identify the ORF encoding for this N-acetylglucosaminidase activity, we assayed also FCE samples of *S. solfataricus* strain PBL2025, a natural deletion mutant of strain 98/2, in which a fragment of about 50 kb of the chromosome containing ~50 ORFs (SSO3004–3050) is missing [33]. Because of the differences between P2, used so far, and 98/2 strains [33], we performed N-acetylglucosaminidase assays also on the latter for a direct comparison. FCE of 98/2 strain showed enzymatic activity units comparable (3.0×10^{-3} U mg^{-1}) to those observed for P2 strain, as confirmed also by the SDS-PAGE activity stained with X-GlcNAc (Fig. 2A). However, interestingly, we could not detect any activity in the PBL2025 strain. These results strongly indicate that the ORF encoding for the N-acetylglucosaminidase activity resides in the chromosome fragment deleted in PBL2025. This region includes a variety of glycoside hydrolases (GH) either already characterized, such as a α-mannosidase (Ssα-man, SSO3006, GH38 [32]), a β-glycosidase (LacS, SSO3019, GH1 [31]), a α-xylosidase (XylS, SSO3022, GH31 [35]), a β-xylosidase/α-L-arabinosidase (XarS, SSO3032, GH3 [43]), or hypothetical, annotated as endo-glucanase (SSO3007, GH5), β-glucuronidase (SSO3036, GH2), α-glucosidase (SSO3037-3038, GH122), and bile-acid β-glucosidase (SSO3039, GH116). Amongst the characterized GHs, LacS and XarS, being specific for β-O-anomeric substrates and belonging to the CAZy families in which N-acetyl-β-hexosaminidases are known, might be able to hydrolyze 4NP-GlcNAc. Thus, purified samples of the two recombinant enzymes were assayed at the same conditions described above and analysed by SDS-PAGE activity stained with X-GlcNAc. Neither of the two enzymes hydrolysed the substrates tested (Fig. 2B and 2C), confirming that the N-acetylglucosaminidase activity was driven by a still unknown GH in *S. solfataricus*.

3.2. Mass spectrometry analysis of N-acetylglucosaminidase partially purified from S. solfataricus

To identify the proteins showing N-acetylglucosaminidase activity, a gel free proteomic approach was carried out. A sample of the final step of the purification (gel filtration chromatography, Table 1) was reduced and alkylated. The protein fraction was then hydrolyzed in solution using trypsin as a proteolytic enzyme and the peptide mixture thus obtained was fractionated by nano LC and analysed by tandem mass spectrometry. The MS/MS spectra were submitted to MASCOT search leading to the identification of at least 47 proteins occurring in the same fraction. Amongst the identified proteins encoded by one of the 50 ORFs (SSO3004-3050) deleted in PBL2025 strain, the highest rankings were observed for the ORFs SSO3006, SSO3032, and SS03039. However, SSO3006 and SSO3032 encoded for a α-mannosidase and XarS, respectively [32,43] and were excluded, whilst SSO3039, showing 26 assigned peptides leading to a sequence coverage of 55% and a MASCOT score of 474, was selected for further characterization.

3.3. Cloning, expression, and characterization of SSO3039

To test whether *sso3039* ORF encodes for an N-acetylglucosaminidase, we cloned the gene in the plasmid pET101/D-TOPO in frame with the C-terminal peptide containing the V5 epitope and a 6xHis tag. After transformation and expression in *E. coli* BL21 star DE3 strain, we performed an easy three-step purification (Table 1), leading to an enzyme >95% pure (Fig. 1D), which showed thermophilic N-acetylglucosaminidase activity.

The characterization of the recombinant SSO3039 revealed, as expected for an enzyme from thermophilic source, an optimal temperature of 70 °C and noticeable stability at 65 and 85 °C (Fig. 3A–C). On the other hand, the optimal pH of 4.0 is rather unexpected from an intracellular enzyme, in fact, pH 4.0 is closer to that of the growing medium rather than to the intracellular one (pH 6.5). Possibly, the enzyme might be involved in the degradation of oligosaccharides after the cell lysis (see Section 4 Conclusions). On the basis of this characterization, the standard assay used in all the following study was performed in 12 mM 4Np-GlcNAc, 50 mM citrate/phosphate buffer, pH 4.0 at 60 °C.

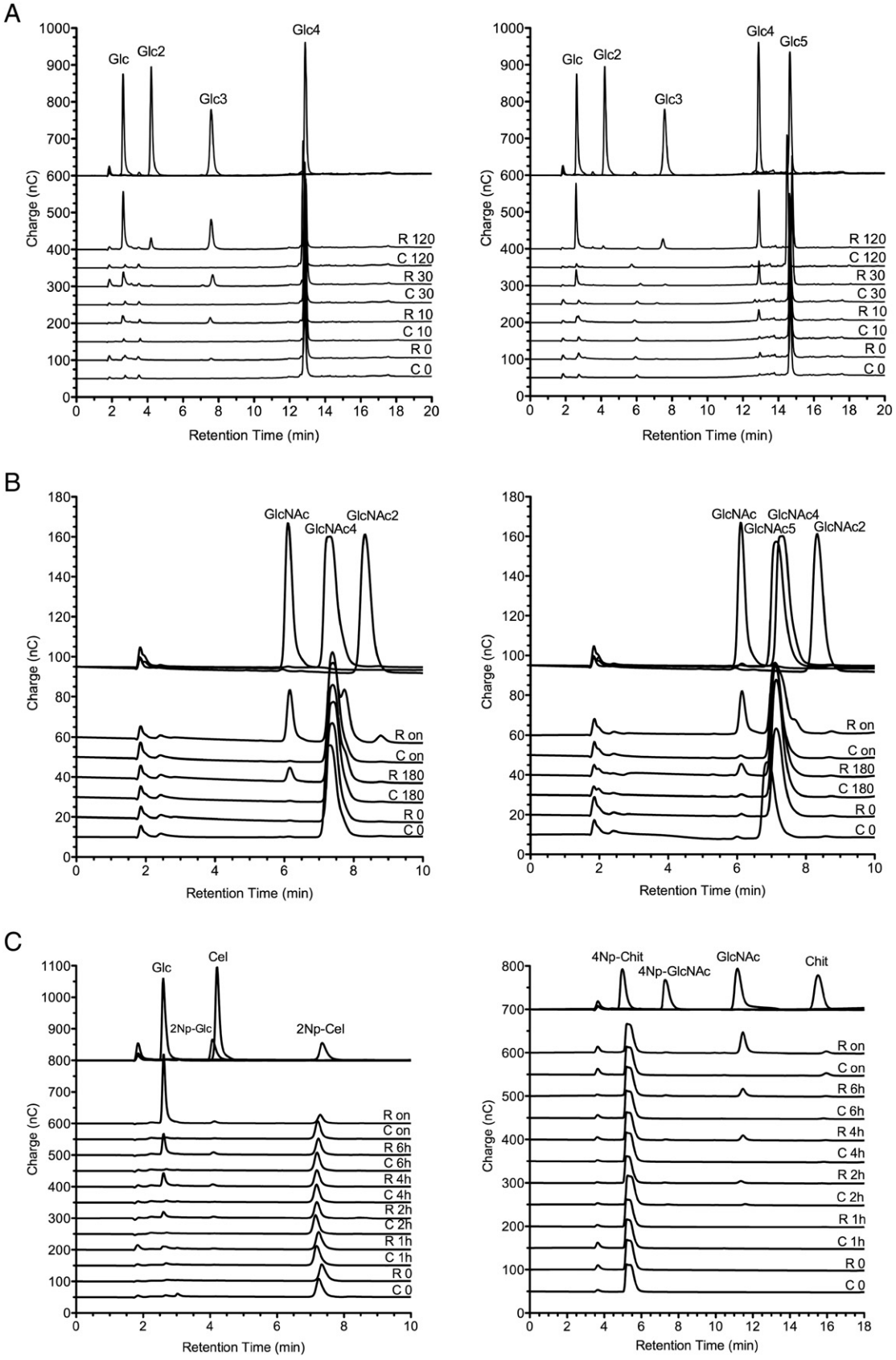

Fig. 4. Substrate specificity and mechanism of action of SSO3039. Activity analysed by High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) on (A) cellotetraose and cellopentaose (left and right panels, respectively); (B) chitotetraose and chitopentaose (left and right pane, respectively); (C) 4Np-cellobioside and -chitobioside (left and right panels, respectively). In all runs, C: negative control; R: reaction with SSO3039.

The molecular mass of the recombinant enzymes in solution was of 313 ± 10 kDa in good agreement with that of the native enzyme (Supplementary Fig. S1); thus, considering that the denaturated monomer is 93.4 kDa, this indicated that SSO3039 is a trimer in solution.

3.4. Substrate specificity

Recombinant SSO3039 confirmed the substrate specificity of the N-acetylglucosaminidase identified in FCE of *S. solfataricus*, with the highest specific activity on 4Np-GlcNAc, followed by aryl-glucosides and -galactosides, whilst the enzyme was only barely active on 4Np-β-Xyl and -β-L-Fuc and inactive on -β-Man (Fig. 3D).

The levels of expression of this gene in *E. coli* were not high, as demonstrated by the final yields (~0.4 mg per litre of *E. coli*) and the 417-purification fold. Purification yields were much lower than those of another GH116 enzyme from *S. solfataricus*, the β-glucosidase/xylosidase SSO1353, which yielded 1.5 mg of pure protein per litre of *E. coli* [38]. On the other hand, it is worth noting that SSO3039 showed a much higher specific activity on 4Np-GlcNAc if compared to that of SSO1353 for 4Np-β-xylopyranoside (7.5 and 0.8 U mg^{-1}, respectively), the preferred substrate of the latter enzyme [38].

The steady state kinetic constant of SSO3039 on 4Np-GlcNAc and 4Np-β-glucopyranoside (4Np-Glc), listed in Table 2, further confirmed the N-acetylglucosaminidase activity, although, as a result of the lower K_M, the specificity constant for 4Np-Glc is about 10-fold higher than that of 4Np-GlcNAc. Interestingly, the kinetic constants on MU-glycosides showed a higher turnover number for MU-Glc, but a 4-fold higher affinity for MU-GlcNAc if compared to 4Np-GlcNAc, resulting in specificity constant for MU-Glc only 2-fold higher than that of MU-GlcNAc. The kinetic properties of SSO3039 on disaccharides, namely cellobiose and *N*,*N*′-diacetylchitobiose, are also described in Table 2: in this case, the k_{cat}/K_M for cellobiose was almost 20-fold higher than that for *N*, *N*′-diacetylchitobiose.

The ability of SSO3039 to hydrolyze longer substrates was tested using β-1,4 oligosaccharides of glucose (Glc) and N-acetylglucosamine (GlcNAc), namely cellotetraose (Glc4), cellopentaose (Glc5), tetra-*N*-acetylchitotetraose (GlcNAc4) and penta-*N*-acetylchitopentaose (GlcNAc5). The enzyme was active on all these substrates: after 10 and 30 min of incubation with Glc5 and Glc4, respectively, we observed the production of glucose and of the oligosaccharide substrates shortened of one unit (cellotriose and cellotetraose from Glc4 and Glc5, respectively) (Fig. 4A). Similarly, SSO3039 hydrolyzed GlcNAc4 and GlcNAc5 producing GlcNAc (Fig. 4B).

The production of monosaccharides observed in the early times of the reaction strongly suggested that the enzyme is an *exo*-glycosidase acting from one of the ends of the substrates. Indeed, SSO3039 released the corresponding monosaccharides (Glc and GlcNAc) after 2 h incubation from both 2Np-β-cellobioside and 4Np-*N*,*N*′-diacetyl-β-chitobioside (2Np-Cel and 4Np-Chit, respectively) (Fig. 4C), unequivocally demonstrating that SSO3039 is an *exo*-glucosidase acting from the non-reducing end of the substrate. The activity on short chitooligosaccharides could suggest the involvement of SSO3039 in chitin degradation. Recently, the product of the ORF STK09390 from *S. tokodaii*, showing 37% identity to the SSO1033 hypothetical protein from *S. solfataricus* strain P2, has been reported to hydrolyze chitin [13]. However, the presence of chitin in the peculiar environment of solfataric fields appears questionable; thus, possibly, these enzymes, together with SSO3039 and its homologue in *S. tokodaii* ST2529, could be involved in the degradation of the glycoproteins and/or the EPS.

One of the peculiarities of GH116 enzymes previously characterized is their sensitivity to competitive inhibitors [38]. In the case of SSO3039, both N-butyldeoxynojirimycin (NB-DNJ) and conduritol β-epoxide (CBE) were inhibitors of the enzyme, but with difference of almost one order of magnitude in IC_{50} (5.15 μM and 9 mM, respectively, see Supplementary Fig. S2). The μM sensitivity to NB-DNJ makes SSO3039 different from both the archaeal GH116 SSO1353, which has mM sensitivity to both inhibitors, and the human non-lysosomal glucosylceramidase also belonging to GH116, which is only sensitive to NB-DNJ [38,44].

Fig. 5. Phylogenetic analysis of GH116. Phylogenetic tree of GH116 family. The three branches corresponding to the subfamilies 1, 2, and 3 are highlighted in blue, green and yellow, respectively (right side). A high-resolution version can be found in the Supplementary material (Supplementary Fig. S9).

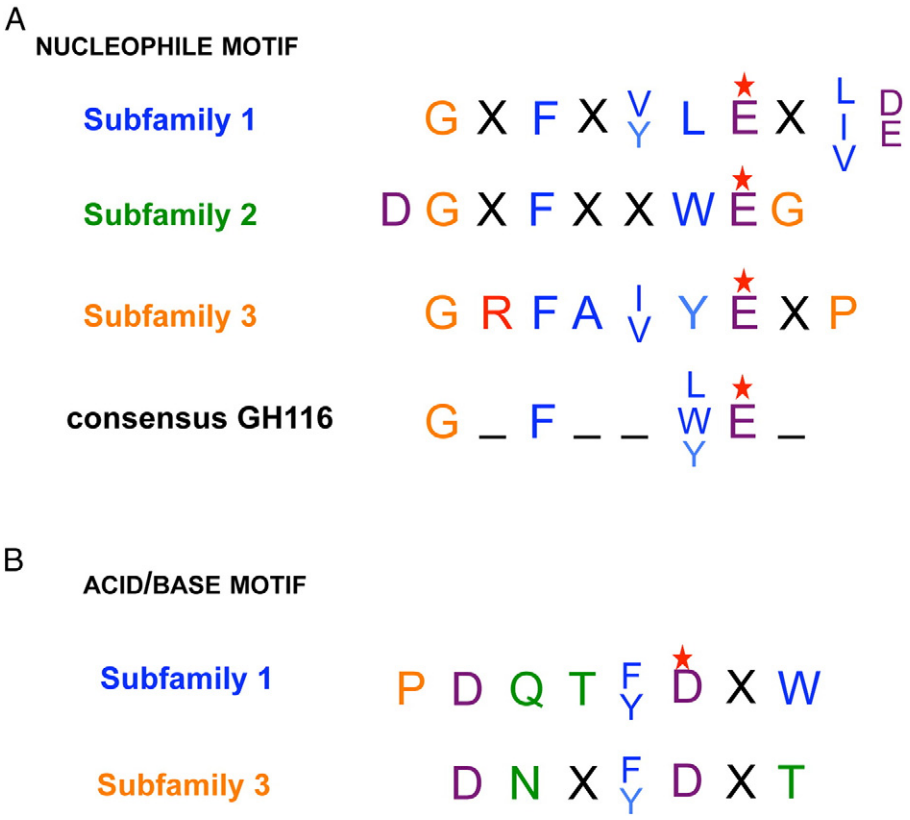

Fig. 6. Comparison of the catalytic motifs of GH116. Catalytic amino acids are highlighted with a red star. Residues are labelled according to the Clustal Omega colouring scheme that is based on the structural characteristics of the residues. In particular, polar amino acids (N, T, S, and Q) are shown in green; hydrophobic (L, V, I, M, F, W, A, and C) in blue; acidic (D, E) in magenta; large aromatic polar (H, Y) in pale blue; basic (K, R) in red; and secondary-structure breaking (G, P) in orange. 'X' indicates the presence in a certain position of residues with different structural characteristics.

3.5. Phylogenetic analysis

The phylogenetic tree reported in Fig. 5 shows that GH116 can be separated in three subfamilies, each of which now has an enzyme characterized in detail: subfamily 1 (highlighted in blue) contains the human non-lysosomal glucosylceramidase (GBA2) [44], subfamily 2 (green) includes SSO3039 described here, and subfamily 3 (yellow) contains the β-glucosidase/xylosidase we described previously [38]. Remarkably, the molecular characterization reported above for the novel β-glucosidase/N-acetylglucosaminidase gives experimental support to this phylogenetic analysis, demonstrating that the three subfamilies are functionally different and have evolved from a common ancestor. Common characteristics of family GH116 are the specificity for β-O-glucosides and the *retaining* reaction mechanism [38]. However, subfamilies 1, 2, and 3, have also specificity for glucosylceramides, N-acetyl-glucosaminides, and xylosides, respectively, and peculiar sensitivity to competitive inhibitors. In fact, GBA2 (subfamily 1) is insensitive to CBE and is inhibited by nM amounts of NB-DNJ [44]. SSO3039 (subfamily 2), as shown here, is sensitive to μM and mM concentrations of NB-DNJ and CBE, respectively, whilst SSO1353 (subfamily 3) shows mM sensitivity to both NB-DNJ and CBE [38].

To verify whether these substrate specificities were driven by primary structural features common to enzymes belonging to each subfamily, we searched the possible presence of conserved subfamily-specific amino acid sequence signatures. The only enzyme in this family for which the essential amino acids have been experimentally identified is SSO1353, in which Glu335 and Asp462 are the nucleophile and acid/base, respectively [38]. The multialignment analysis of the amino acid sequences of all GH116 members against SSO1353, indeed confirms that these two residues are invariant in the whole family, strongly fostering their essential role in GH116 (see the complete alignments in the Supplementary Fig. S3–S8) and allowing us to predict that in SSO3039 Glu321 and Asp539 are the nucleophile and the acid/base residues, respectively.

To identify consensus sequences in the region of the nucleophile and the acid/base, respectively, in the three subfamilies of GH116, we prepared three different data sets. The one for subfamilies 1 and 3, including the 103 and 34 members shown in Fig. 5, respectively, revealed clear consensus sequences surrounding the catalytic residues (Fig. 6 and Supplementary Figs. S3, S4, S7, and S8). The data set for subfamily 2, including the 30 members in Fig. 5, produced reliable amino acid sequence alignments only in the region of the nucleophile (Fig. 6A and Supplementary Fig. S5). Instead, the acid/base Asp539 identified with the robust multialignment of all members of GH116, was mis-aligned when a sub-set of sequences was used (Supplementary Fig. S6). These observations indicate that the acid/base in subfamily 2 of GH116 is placed in a rather variable region and, therefore, we prefer to omit the consensus produced by the programme Clustal Omega with this data set.

The comparison of the sequence motifs of the three subfamilies is described in Fig. 6. In subfamily 1, which includes GH116 enzymes from tunicates, plants and mammals, the two sequence motifs are rather well conserved confirming the prediction made previously that in GBA2 the nucleophile and the acid/base of the reaction are Glu527 and Asp677, respectively [38]. Clear conserved motifs could be also observed in subfamily 3 and in the region of the nucleophile of subfamily 2, both grouping enzymes from Bacteria and Archaea. Clearly, the region surrounding the nucleophile is better conserved than that of the acid/base, with invariant Gly and Phe residues and a highly hydrophobic region preceding the catalytic Glu. By contrast, the acid/base residues are located in a more variable region, which prevented the reliable definition of a consensus sequence. However, the differences amongst the

motifs of the three subfamilies support the experimental observation that the enzymes from the three GH116 subfamilies have peculiar substrate specificities and competitive inhibitor sensitivities. The experimental confirmation of these observations goes beyond the aims of the present work and future characterizations of other GH116 members and the probing of the amino acids involved in the substrate specificity of these enzymes will shed light on their structure/function relationship.

4. Conclusions

We described here the first characterization of an archaeal bifunctional β-glucosidase/exo-N-acetylglucosaminidase, along with the division of family GH116 into three subfamilies. The precise *in-vivo* function of this enzyme cannot be easily predicted from the data presented here, possibly, it could be involved in the degradation and recycling of the glycans decorating glycoproteins and/or the EPS. Further experiments are needed to clarify these aspects, however, the detailed biochemical characterization of enzymes from natural source is of crucial importance to facilitate the discovery of enzymes with similar properties in other organisms and to improve functional predictions during genome annotations.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.09.022.

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