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Action of a GH115 α-glucuronidase from *Amphibacillus xylanus* at alkaline condition promotes release of 4-*O*-methylglucopyranosyluronic acid from glucuronoxylan and arabinoglucuronoxylan

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ABSTRACT

Glucuronic acid and/or 4-*O*-methyl-glucuronic acid (GlcA/MeGlcA) are substituents of the main xylans present in hardwoods, conifers, and many cereal grains. α-Glucuronidases from glycoside hydrolase family GH115 can target GlcA/MeGlcA from both internally and terminally substituted regions of xylans. The current study describes the first GH115 α-glucuronidase, AxyAgu115A, from the alkaliphilic organism *Amphilbacillus xylanus*. AxyAgu115A was active in a wide pH range, and demonstrated better performance in alkaline condition compared to other characterized GH115 α-glucuronidases, which generally show optimal activity in acidic conditions. Specifically, its relative activity between pH 5.0 and pH 8.5 was above 80%, and was 35% of maximum at pH 10.5; although the enzyme lost 30% and 80% relative residual activity after 24-h pre-incubation at pH 9 and pH 10, respectively. AxyAgu115A was also similarly active towards glucuronoxylan as well as comparatively complex xylans such as spruce arabinoglucurunoxylan. Accommodation of complex xylans was supported by docking analyses that predicted accessibility of AxyAgu115A to branched xylo-oligosaccharides. MeGlcA release by AxyAgu115A from each xylan sample was increased by up to 30% by performing the reaction at pH 11.0 rather than pH 4.0, revealing applied benefits of AxyAgu115A for xylan recovery and processing.

1. Introduction

As the second most abundant plant polysaccharide next to cellulose [1], xylans are being developed for a broad range of applications, from a source of sugars for biofuels, sweeteners, and prebiotics, to applications of the biopolymer in hydrogels, packaging, surfactants, and coatings [2,3]. Because the functional properties of xylans are affected by their side-chain chemistry, so-called accessory hemicellulases have been used to modify xylan branching structures with the ultimate goal of making xylan-derived polymers with desired characteristics [4–8]. Glucuronic acid and/or 4-*O*-methyl-glucuronic acid (GlcA/MeGlcA) are typical substituents that are α-(1 → 2)-linked to xylans from hardwoods, conifers, cereals and grasses [9–11]. α-Glucuronidases with ability to hydrolyze linkages between GlcA/MeGlcA and D-xylopyranosyl (Xyl*p*) residues were reported in glycoside hydrolase (GH) families GH67 and GH115 of the carbohydrate active enzyme (CAZyme) classification system (www.cazy.org) [12–17]. Only those enzymes belonging to family GH115, however, were shown to effectively target polymeric substrates due to their ability to cleave both internally and terminally substituted GlcA/MeGlcA residues [13,14,16].

So far, seven GH115 α-glucuronidases of more than 485 predicted sequences have been characterized, including four bacterial enzymes (BoAgu115A [14], BtGH115A [18], SdeAgu115A [17] and Pjdr2Agu115A [19]), as well as three fungal enzymes (SpAgu115A [20], ScAgu115A [13] and PsAgu115A [16,21]). With the exception of BtGH115A which targets MeGlcA residues from arabinogalactan, all other enzymes from family GH115 that have been characterized to date act upon glucuronoxylan. Protein structures have also been solved for BoAgu115A, BtGH115A, and SdeAgu115A, where both BoAgu115A and BtGH115A adopt a four-domain architecture and SdeAgu115A as a five-domain architecture [14,17,18].

It was shown that xylans with more accessible surface area could facilitate xylanase activity [22]. Since high molecular weight glucuronoxylans are better dispersed in alkaline conditions due to electrostatic repulsion between charged polysaccharides, it is conceivable that alkaline-tolerant GH115 enzymes would permit more extensive release

Abbreviations: GlcA/MeGlcA, glucuronic acid and/or 4-*O*-methyl-glucuronic acid; Ara*f*, arabinofuranosyl; Xyl*p*, D-xylopyranosyl; AGX, arabinoglucuronoxylan; OSX, oat spelt xylan; BEX, beechwood xylan

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of GlcA/MeGlcA from high concentrations of glucuronoxylan when prepared as an alkaline solution. *Amphibacillus xylanus* strains were previously isolated from an alkaline compost [23]. The genome of *A. xylanus* NBRC 15112 (GenBank: AP012050.1; Taxonomy ID: 698758) was released in 2013, and the type strain (Ep01^T, also known as JCM 7361 and NBRC 15112) was confirmed to utilize xylan from oat spelt and larchwood, and to grow between pH 8 and pH 10 [23]. In the current study, a recombinantly expressed GH115 α-glucuronidase from *A. xylanus* (AxyAgu115A) was biochemically characterized and shown to display the highest relative activity in alkaline condition among all other characterized GH115 α-glucuronidases with known pH profiles. The high pH tolerance of AxyAgu115A was then correlated to its improved ability to hydrolyze complex xylans such as oat spelt xylan (OSX) and spruce arabinoglucuronoxylan (AGX).

2. Materials and methods

2.1. Materials

Spruce arabinoglucuronoxylan was kindly provided by Professor P. Gatenholm and Professor G. Toriz, and it was produced as previously described [8]. D-Glucuronic acid assay kit was purchased from Megazyme. SdeAgu115A and MeGlcA-substituted xylo-oligosaccharides ($U^{4m2}XX$ and $XU^{4m2}XX$) were produced in our laboratory based on previously published methods [17]. All other chemicals were analytical grade and obtained from Sigma Aldrich or Fisher Scientific.

2.2. Bioinformatic analyses

Amino acid sequences from the GH115 family were retrieved from the CAZy database (July 15th, 2016). Curation was performed by removing sequences that were shorter than 200 amino acid residues or lacked Domain B shown in other GH115 members to potentially comprise catalytic amino acids [17]. In total, 302 sequences were collected and aligned using MUSCLE [24], and a phylogenetic tree was created using Tree Builder and the neighbor-joining method of Geneious (Geneious 8.1.8).

Protein structure homology modeling was performed using the SWISS-MODEL server (http://swissmodel.expasy.org/), in which the 3D model of AxyAgu115A was built using the X-ray structure of SdeAgu115A (PDB ID: 4ZMH) as a template [25]. The structural models of $U^{4m2}XX$ and $XU^{4m2}XX$ were drawn based on previously reported structural information [26,27], while that of $XU^{4m2}XXA_3X$, where an arabinopyranose side group is two xylose units away from a MeGlcA side group, was drawn based on the NMR analysis of spruce arabinoglucuronoxylan [11]. AutodockTools v1.5.2 on Python v2.5 (http://autodock.scripps.edu/) was used to adjust the torsion of oligosaccharides, position polar hydrogen atoms within proteins, and to prepare the grid box. Docking simulation was conducted using Autodock Vina v1.1.2 (http://http://vina.scripps.edu/). Protein structure figures were generated by PyMol (The PyMOL Molecular Graphics System. DeLano Scientific; San Carlos, CA, USA: 2002).

2.3. DNA manipulation

The gene encoding AxyAgu115A (GenBank ID: BAM48432.1) was codon optimized for expression in *Escherichia coli* using the OptimumGene™ algorithm, and the optimized gene was synthesized and provided in pUC57 vector by GenScript. The *A. xylanus aguA* gene was amplified from the pUC57 vector using CloneAmp™ HIFI PCR Premix (Clontech) and primers that are compatible with infusion cloning. The nucleotide sequences of the forward and reverse primers for PCR amplification were 5′-TTGTATTTCCAGGGCATGGACTTTACCCTGAACCAAG-3′ and 5′-CAAGCTTCGTCATCATTAGCGACCCACATAGTAGGTTTC-3′, respectively. The purified PCR product was transferred to the p15Tv-L expression vector (GenBank accession EF456736) using the infusion cloning kit from Clontech (California, USA), generating p15Tv-L_AxyAgu115A.

2.4. Expression and purification of α-glucuronidase AxyAgu115A

Production of AxyAgu115A in *E. coli* BL21(λDE3) CodonPlus was carried out at 37 °C in 1L Luria-Bertani medium containing 500 mM D-sorbitol, 2.5 mM glycine betaine, 34 μg mL^{-1} chloramphenicol and 100 μg mL^{-1} ampicillin. When the OD_{600nm} of the cultivation reached 0.6, the cultivation was cooled to 15 °C before addition of isopropyl β-D-thiogalactopyranoside to 0.5 mM. Following 16 h of induction, cells were harvested by centrifugation at 6000 × *g* for 30 min, suspended in 30 mL of protein binding buffer (300 mM NaCl, 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 7.0, 5% glycerol, and 5 mM imidazole), and then sonicated at 100 Amps for 15 min (10 s for pulse-on and 4 s for pulse-off). Removal of cell debris was achieved by centrifugation at 17,500 × *g* for 20 min, followed by filtration through a 0.45-μm filter. The clarified supernatant was then incubated with 5 mL NiNTA resin for 2 h at 4 °C, before washing with 800 mL of washing buffer (300 mM NaCl, 50 mM HEPES pH 7.0, 5% (v/v) glycerol, and 50 mM imidazole). A total of 45 mL of elution buffer (300 mM NaCl, 50 mM HEPES pH 7.0, 5% v/v glycerol, and 250 mM imidazole) was then used to elute the recombinant protein as 3 mL fractions. Active fractions were pooled, exchanged to 25 mM HEPES buffer (pH 7.0) containing 300 mM NaCl using a Bio-Gel P10 column, and then concentrated using Amicon centrifugal units (NMWL, 10 kDa, Millipore). Protein concentration was determined using the Bradford assay and bovine serum albumin as a standard; protein purity was assessed by SDS-PAGE. Aliquots of purified protein were flash frozen in liquid nitrogen and stored at −80 °C.

2.5. Enzyme assay

AxyAgu115A activity was measured using the D-glucuronic acid assay kit from Megazyme. The standard reaction was performed for 5 min at 55 °C and were initiated by adding 5 μL enzyme (final concentration of 5 ng/μL) to 20 μL of substrate (final concentration of 1.0% (w/v)) in 200 mM universal buffer (67 mM H_3BO_3, 67 mM H_3PO_4, 67 mM CH_3COOH; adjusted to pH 7.0 using sodium hydroxide). Enzyme reactions were stopped by heating at 100 °C for 10 min. Kinetic parameters were obtained using 0.05–38.4 mM $U^{4m2}XX$, 0.05–6.37 mM $XU^{4m2}XX$, 0.1–80 mg/mL beechwood xylan and 0.1–80 mg/mL spruce arabinoglucuronoxylan. Kinetic parameters were calculated using GraphPad 5.0 (GraphPad Software, USA).

2.6. Effect of temperature, pH, and metal ions

The thermostability of AxyAgu115A was evaluated by incubating the enzyme at pH 7.0 for up to 24 h at 40 °C, 45 °C, 50 °C, 55 °C, 60 °C and 65 °C. Following incubation, the enzyme was quickly cooled on ice, and the residual activity was measured by transferring the heat-treated enzyme (final concentration was 5 ng/μL) to a reaction mixture comprising 200 mM universal buffer (pH 7.0), 1.0% (w/v) beechwood xylan; following 5 min at 55 °C, reaction products were quantified using the D-glucuronic acid assay kit. The pH optimum of AxyAgu115A was determined by measuring activity as described above, this time at 55 °C in 200 mM universal buffer at pH 3.0–11.5. To determine pH stability, residual AxyAgu115A activity was measured after 24 h-incubation at 4 °C in 200 mM universal buffer with pH range from 3.5 to 10.5. The effect of metal ions was tested based on a previously published method [28]. Briefly, 150 μg AxyAgu115A was separately pre-incubated with Chelex 100 (Sigma) in 25 mM HEPES buffer at pH 7.0 for 20 min to remove metal ions, then Chelex 100 was removed using a 0.2-μm filter. Activities of the apoenzyme were determined with and without supplementation with 1 mM $MgCl_2$, $CaCl_2$, $FeCl_3$, $CuSO_4$, $NiCl_2$, $AgNO_3$, $SrCl_2$ and $ZnCl_2$. To test the effect of salt on enzyme activity, AxyAgu115A (final concentration of 5 ng/μL) was incubated at 55 °C for