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Research Article

A Novel Thermostable GH51 A-L-Arabinofuranosidase from *Caldicellulosiruptor Lactoaceticus* and the Application for Natural Hemicellulose Degradation

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2. Keywords

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1. Abstract

Improving the hydrolytic performance of xylanolytic enzymes on complicated xylan is of great importance in lignocellulosic biomassbased fermentation industry. Employing a de-branching enzymeα-L-arabinofuranosidase (EC 3.2.1.55)in conjunction with backbone depolymerizing enzyme endo-β-1,4-xylanase (EC 3.2.1.8) offers a way to address this problem.In present study,a glycoside hydrolase family 51α-L-arabinofuranosidase Abf51A from extremely thermophilic bacterium Caldicellulosiruptor lactoaceticuswas cloned, expressed and characterized in Escherichia coli.The recombinant Abf51A existed as a homohexamer in solution, which showed optimal activity at 80°C and pH 5.5, and had long-term thermostability at 75°C and 80°C after 12 h incubation. Abf51A was most active on p-nitrophenyl-α-L-arabinofuranoside with Km and Vmax values reaching 0.52 ± 0.05 mM and 2981.00 ± 79.65 μmol/min/mg, respectively. In addition, it also displayed minor activity with p-nitrophenyl-β-D-xylopyranoside. Polymeric substrates screening revealed that Abf51A released arabinose from arabinoxylan (oat spelt xylan) and glucuronoarabinoxylan (sugarcane xylan and corncob xylan). Moreover, Abf51A showed obvious synergism with endo-β-1,4-xylanase Xyn10Aon both arabinoxylan and glucuronoarabinoxylan degradation, suggesting an significant contribution of Abf51A in arabinose-substituted xylansutilization. The thermostability, hydrolytic properties and synergism action made Abf51A a promising candidate in the production of fermentable pentoses from arabinose-containing xylans.

3. Introduction

Xylan, asthemost abundanthemicellulosic constituent of plant cell walls, has being considered as an abundant source of fermentable pentose (mainly xylose and arabinose) for biofuels and bio-chemicals. Generally, it consisted of a linear backbone of β-1,4-linked D-xylopyranosyl units which can be degraded by core enzymes endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) (Dodd and Cann 2009). Xylan is usually linked with various side-chain substitutions to its main chain, such as L-arabinofuranosyl, acetyl, feruloyl and glucuronic acids residues. Hence xylans are grouped into linear homoxylan, arabinoxylan (AX), glucuronoxylan, glucuronoarabinoxylan (GAX) and heteroxylan based on these substituents on the backbone [1]. However, the presence of these side chains on xylan can strongly restrict the action of endo-β-1,4-xylanase and β-xylosidase, thus preventing the complete breakdown of the polymer to its basic units [2]. Therefore, among the xylanolytic enzyme system, de-branching enzymes such as α-L-arabinofuranosidase (EC 3.2.1.55, AFase),α-glucuronidase (EC 3.2.1.131) and acetylxylan esterase(EC 3.1.1.72) are required for the synergistically enzymatic action on polymeric xylan. Currently, these side chain-removing accessory enzymes have been used collaboratively with other lignocellulose-decomposing en-

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zymes in biotechnological processes [3]. AFases are hemicellulases that cleave the 1-2, 1-3 and 1-5 glycosidic bond at the non-reducing terminal ends between L-arabinofuranoside substituents and various poly- and oligo-saccharides. These enzymes are part of glycoside hydrolases (GHs) responsible for the degradation of arabinose-existing hemicelluloses such as L-arabinan, AX and arabinogalactan [4]. Based on the amino acid sequence similarities and hydrophobic cluster analysis, AFases have been classified into GH families 3, 43, 51, 54 and 62, with families GH51, 43 and 62 as the main families (CAZY; http://www.cazy.org/). To date, GH51 is the largest characterized family with a majority of them being of bacterial origin. Structure data obtained for several members of this family confirm that GH51 AFases process a catalytic domain with $(\beta/\alpha)_8$-barrel architecture, and additionally uptake a unknown function domain composed of β-sandwich at the C-terminal. Besides, GH51 enzymes preform glycosidic bond cleavage *via* a retaining mechanism with two conserved glutamate residues acting as acid/base and nucleophile.So far, the majority of characterized GH51 AFases showed only exo-arabinanaseactivity with arabino-oligosaccharides and/or L-arabinose containing heteropolymers such as arabinan and AX [5]. A fewof GH51 AFases also display endo-β-D-xylanase activity [6]. A finely planned cocktail of enzymes, containing not only cellulases but also xylanases, is required for efficient fermentable sugars yield from the polysaccharide components of lignocellulosic biomass.In this respect, thermostable enzymes process a number of important advantages compared with their mesophilic counterparts in the optimization of biorefinery-scale lignocellulosic depolymerization [7]. Various bacteria, archaea, fungi and plants have received attentions as sources for thermostable xylanolytic enzymes. Thermophilic microbes provide convenient approaches to obtain thermostable enzymes active on lignocellulose.*Caldicellulosiruptor lactoaceticus* 6A, an extremely thermophilic plant biomass degrading bacterium, grows on natural xylan as sole carbon source [8]. In our previous studies on glycoside hydrolases for de-branched xylan degradation from this bacterium, a thermophilic endo-β-1,4-xylanase Xyn10A with good thermostability has been reported [9]. Further insight into xylanolytic enzymes other than endo-β-1,4-xylanase of this bacterium would provide not only new exploration to the entire mechanisms for xylan utilization of *C. lactoaceticus*, but also approach for biomass conversionat extreme temperature.In present study, the geneencodingAFase Abf51A was identified and cloned from the genome of *C. lactoaceticus* 6A, then expressed in *E. coli*and biochemicallycharacterized. Additionally, the synergistically hydrolytic function of AFase together with endo-β-1,4-xylanaseon heteroxylan hydrolysis wasalso studied.

4. Materials and Methods

4.1. Strains, plasmids and chemicals

*C. lactoaceticus*6A (= DSM 9545) was obtained from DSMZ (Braunschweig, Germany). *Escherichia coli* Top10 (TianGen, China) and plasmid pET-28b (Novagen, USA) were used for gene cloning, and *E. coli* Rosetta (DE3) (Novagen, USA) was used for protein expression and purification. *p*-nitrophenyl-α-L-arabinofuranoside (*p*NP-Af),*p*-nitrophenyl acetate (*p*NP-Ace),*p*-nitrophenyl-α-D-xylopyranoside (*p*NP-α-Xyl),*p*-nitrophenyl- β-D-xylopyranoside (*p*NP-β-Xyl),*p*-nitrophenyl-β-D-cellobioside (*p*NP-C) and *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glu) were purchased from Sigma-Aldrich (St. Luis, USA). Oat spelt xylan(OSX) was purchased from Hualan Chemical Technology Co., Ltd. (Shanghai, China).Corncob xylan (CCX) and sugarcane xylan (SCX) was presented by ProfessorHourui Zhang from Guangxi Institute of Botany, Chinese Academy of Sciences. D-xylose, L-arabinose, locust bean gum, soluble starch, Avicel, carboxymethyl cellulose (CMC), *p*-nitrophenol, and other chemicals were obtained from Kepujia Reagent Co. (Beijing, China). All other chemicals were of analytical class unless otherwise stated.

4.2. Genomic DNA extraction and amplification

C. lactoaceticus 6A was cultivated in DSM 671 medium at 70°C for 4 d under anaerobic conditions. The genomic DNA of C. lactoaceticus 6A was isolated using a TIANamp Bacteria DNA Kit (TianGen, Beijing, China). The gene abf51A encoding a hypothetical AFase [GenBank: AEM73175.1] was predicted and amplified based on the whole genome [10]. PCR reaction was performed with Pfu DNA polymerase (TianGen) and the procedure was as follows: 95°C 5 min; 30 cycles of 95°C 30 s, 55°C 1 min, and 72°C 1 min; final 72°C 5 min. Target PCR products were then purified using TIAN Gel Midi Purification Kit (TianGen) and stored at -20°C until use.

4.3 Construction and sequencing of the expression vector

The purified PCR products of *abf*51A amplification were then digested with T4 DNA polymerase (Takara, Dalian, China) and inserted into pET-28b EK/LIC vector. The sub-cloned plasmid was then transferred into *E. coli* Top10 competent cells by heat shock and grown overnight on Lysogeny Broth (LB) agar plate containing 50 μg/mL kanamycin at 37°C. Positive cloning junctions were confirmed by colony PCR and DNA sequencing with T7 primers from both strands.

4.4. Expression, purification and gel filtration chromatography

The recombinant plasmid was isolated using TIANprep Mini Plasmid Kit (TianGen) and transferred into *E. coli* Rosetta (DE3)

competent cells by heat shock and grown overnight on LB agar plates containing 50 μg/mL kanamycin and 34 μg/mL chloramphenicol at 37°C. One single colony was picked out as seed culture and grown overnight in 5 mL LB liquid medium containing 50 μg/mL kanamycin and 34 μg/mL chloramphenicol at 37°C on a rotary shaker (220 rpm). The culture was induced with 0.5 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) after the optical density reached 0.6 at 600 nm for an additional 6 h at 37°C. The cells were harvested by centrifugation (4000 rpm for 20 min at 4°C), and resuspended in 20 mL binding buffer (50 mM Tris-HCl, pH7.5, 300 mM NaCl). Then the cell suspension was lysed by sonication on ice and cell debris was removed by centrifugation (12000 rpm for 20 min at 4°C). After then, the supernatant was heat-treated at 50°C for 30 min and denatured proteins were removed by centrifugation (12000 rpm for 20 min at 4°C). The resulting supernatant was then loaded onto a Ni-NTA affinity column (National Engineering Research Centre for Biotechnology (NERCB), Beijing, China), which was pre-equilibrated with binding buffer for five times.Subsequently,the column was washed with binding buffer for three times and eluted with elution buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 150 mM Imidazole). Hereafter, the molecular mass and oligomeric state were further determined using a 1 × 33 cm^3Superdex 200 exclusion column (Mengyimei Bio-Tech Co., Ltd. Beijing, China) using citrate buffer (50 mM sodium citrate, 150 mM NaCl, pH 6.0) as the mobile phase. Approximate 500 μL sample was loaded onto the column at a flow rate of 0.5 mL/min using Huxi chromatographic separation system (Shanghai Huxi analysis instrument factory, Co. LTD, China). At the same time, the homogeneity of the target protein was checked by sodium dodecyl sulfate-polycrymide gel electrophoresis (SDS-PAGE) in accordance with the technique described by Laemmli [11].

4.5. Enzyme assay and protein determination

The activity of AFase was measured using *p*NP-Af as the substrate as described by Qiaojuan Yan [12] with slight modifications. The reaction mixture, contained diluted enzyme, 1 mM *p*NP-Af and 50 mM citrate buffer (pH 5.5) in a 50 μL reaction volume, was incubated at80°C for 3 min. The reaction was then stopped by adding 100 μL of 1 M Na_2CO_3 and the number of *p*-nitrophenol generated was measured at 400 nm. A standard curve was prepared with*p*-nitrophenol as a standard. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute under the standard assay conditions. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard. All of the assays were performed in triplicate.

4.6. Biochemical characterization of the recombinant enzyme

Rapid screening of Abf51A activity was firstly conducted with various aryl-monosaccharides including *p*NP-Af, *p*NP-Ace, *p*NP-β-Xyl, *p*NP-α-Xyl, *p*NP-C and *p*NP-Glu. Besides, the substrate specificity was also detected with 1% (*w/v*) polysaccharides includingOSX, CCX, SCX, locust bean gum, soluble starch, Avicel and CMC. One unit of Abf51A activity towards polysaccharides was defined as the amount of enzyme required to release 1 μmol of L-arabinose equivalent per minute under the standard assay conditions.The optimum temperature for Abf51A activity was measured in a temperature range of 40-95°Cand pH 6.5 with *p*NP-Af as substrate as described above. In addition, the optimum pH for Abf51A activity was determined by using 50 mM phosphate-citrate buffers (pH 4.0-8.5) at 75°C with *p*NP-Af as substrate. To detect the thermostability of the enzyme, purified Abf51A were incubated in 50 mM phosphate-citrate buffer (pH 6.0) at 75, 80 and 85°C for different time periods in the absence of substrate. The residual activity of Abf51Aunder optimal conditions was assayed at the time period of 0.5, 1, 2, 4, 6 and 12 h with *p*NP-Af as substrate. All of the experiments were performed in triplicate. Moreover, the kinetic parametersK_m and V_{max} for Abf51A were determined by incubating enzyme with *p*NP-Af at various concentrations (0.01-5.0 mM) under optimal conditions and calculated from a Lineweaver-Burk plot using GraphPad Prism version 6.0 for Windows (GraphPad Software Inc., USA).

4.7. Synergistic hydrolysis of xylan

To investigate the synergistic effect of Abf51A in combination with β-1,4- xylanase, the GH family 10 xylanase Xyn10A from *C. lactoaceticus* 6A was purified as previously described [13]. The reaction mixture (1 mL) contained 1% (*w/v*) substrates (OSX, CCX and SCX), 50 mM citrate buffer (pH 6.0), and 25 μg purified enzyme (Abf51A and Xyn10A, either alone or incombination). Each of the control reaction was performed under the same experimental condition except adding corresponding heat-denatured enzymes. After incubation at 80°C for 24 h, the reaction was stopped by boiling for 10 min, and centrifuged at 12000 rpm for 10 min. The amount of reducing sugars was determined using *para*-hydroxybenzoic acid hydrazide (PHBAH) method with D-xylose as a standard [14]. At the same time, the released D-xylose and L-arabinosewere determined by high performance liquid chromatography (HPLC) using a Hi-Plex Ca column (300 × 7.7 mm, Agilent Technologies, United Kingdom) and a refractive index detector (LC-20AT, Shimadzu Corp., Japan). The column temperature was maintained at 85°C with Milli-Q filtered HPLC grade water as mobile phase at a flow rate of 0.6 mL/min. The injection volume was 10 μL with D-xylose and L-arabinose as standards. All the assays were performed for three times.

5. Results

5.1. Sequence analysis and phylogenic analysis

Through *C. lactoaceticus* genome sequence analysis, Calla_0521was annotated as a putative AFase (Abf51A). Abf51A was predicted to have no signal peptide indicating it was an intracellular enzyme. The *abf*51A gene was amplified to encode 505 amino acid residues, and the calculated molecular weight (Mw)and deduced theoretical isoelectric point (p*I*) of the protein were 57.87 kDa and 5.83, respectively. Abf51A showed the highest (99%) identity with AFases from *Caldicellulosiruptor acetigenus*[GenBank: WP_029228247.1] and *Caldicellulosiruptor kristjanssonii*[GenBank: WP_013432406.1], and higher(92-98%) identitywith other predicted AFases from *Caldicellulosiruptor* sp. Besides, Abf51A exhibited 70% identity with AFasefrom thermophilic bacteria *Dictyoglomusthermophilum*[GenBank: WP_012548731.1], *Dictyoglomusturgidum* [GenBank: WP_012584082.1] and *Mahellaaustraliensis* [GenBank: WP_013779842.1]. Moreover, to gain further insight into the evolutionary relationship between GH51 AFases, phylogenetic tree was constructed using 18 protein sequences of GH51 AFasesby Neighbor-Joining method **(Figure. 1)**. The results revealed that Abf51A was clustered with GH51 AFase from *Caldicellulosiruptor saccharolyticus* DSM 8903 [GenBank: ABP67153.1], then located closer with GH51 AFases from *Paenibacillus* sp. Besides, amino acids sequence blast analysis revealed that Abf51A contained an Alpha-L-AF-C superfamily motif (291-494 amino acids) which had significant similarity with other GH51 AFases. Aligning the Abf51A sequence with several GH51 AFases revealed that they shared the conserved catalytic residues, that was Glu173 acting as the acid/base, and Glu292 acting as the catalytic nucleophile **(Figure. 2)**.

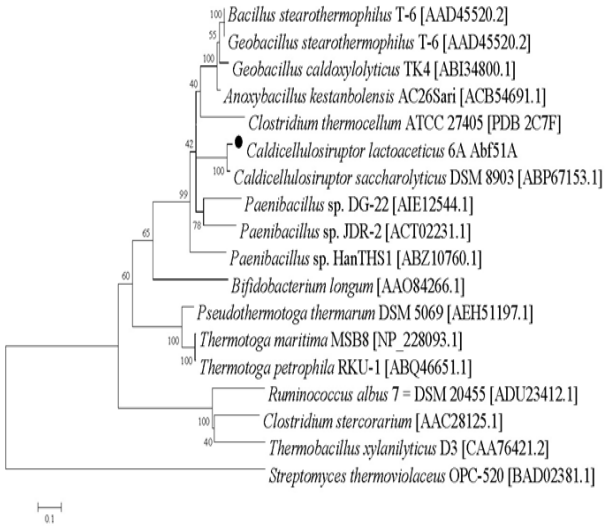

Figure 1: Phylogenetic tree analysis of Abf51A.The Neighbor-Joining tree was constructed using MEGA 5.05, bootstrap value was 1000 replicates, and GenBank accession numbers of each protein sequence were listed after scientific names.

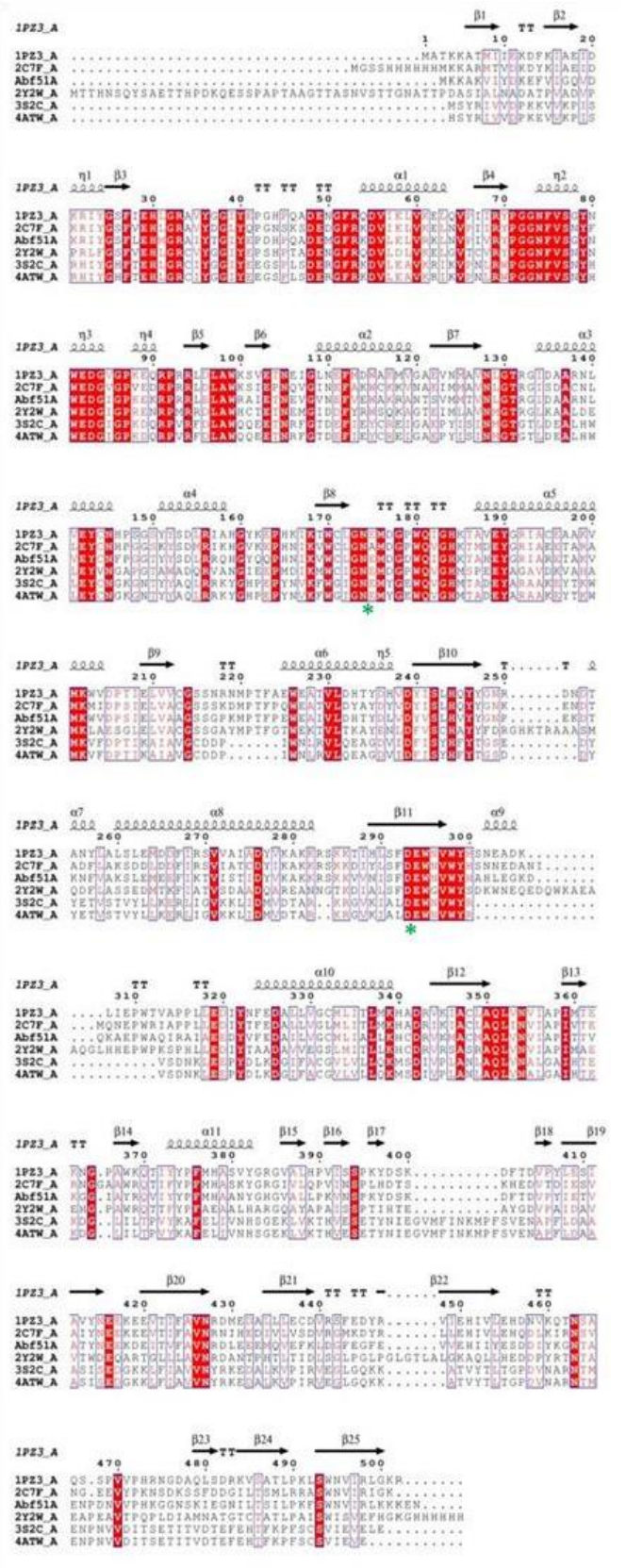

Figure 2: Multiple amino acid sequence alignment of Abf51A.Sequence alignment was computed on http://www.genome.jp/tools/clustalw/ and depicted by ESPrit 3.0: http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi, respectively. Identical and similar amino acid residues were enclosed in black boxes and open boxes, respectively. The catalytic residues Glu173 and Glu292 of Abf51A were marked by black stars below the conserved sequences. The alignment for Abf51A included GH51 AFases from *Geobacillus stearothermophilus* T6 [PDB: 1PZ3_A], *Ruminiclostridiumthermocellum* [PDB: 2C7F_A], *Bifidobacteriumlongum* [PDB: 2Y2W_A], *Thermotoga petrophila* RKU-1 [PDB: 3S2C_A]and *Thermotoga maritima* MSB8 [PDB: 4ATW_A].

5.2. Structure analysis

The structure of Abf51A was built by homology modeling from Swiss Model server (Figure. 3). The closest monomer homolog was GH51 AFase from *G. stearothermophilus* T6 [PDB: 1PZ3_A], with which Abf51A shares 65.1% identity and for which the structure-function relationship was defined. The overalltwo domain structure of Abf51A monomeric subunit composed oftwo domains: a $(\beta/\alpha)_8$ barrel and a C-terminal 12-stranded β-sandwichwith a jelly-roll topology.The homology model of Abf51A also confirmed the putative role of acid/base catalyst Glu173 and nucleophile residue Glu292.

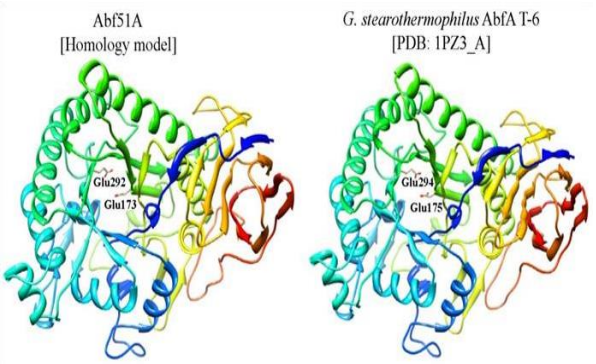

Figure 3: Structural model of Arf51A.Structure was created by Swiss-model and processed by Chimera 1.6.2. The structure of Abf51A was built with the GH51 AFase from G. stearothermophilus T6 [PDB: 1PZ3_A] as template. The catalytic residues were shown as yellow sticks.

5.3. Purification and gel filtration chromatography property

For functional analysis of the recombinant enzyme, Abf51A wascloned into pET-28b vector and expressed in *E. coli* Rosetta (DE3). The recombinant protein from the crude extract was purified by Ni-affinity chromatography and further determined through Superdex 200 gel filtration. Recombinant Abf51A displayed as a single band with anMw of about 57 kDa after SDS-PAGE analysis, which was consist with the predicted Mw based on the amino acid sequence **(Figure.4a)**. Size exclusion chromatography revealed that Abf51A eluted as a single peak corresponding to Mw of340 kDa, suggesting that Abf51 existed as a homohexamerin solution **(Figure.4b)**.

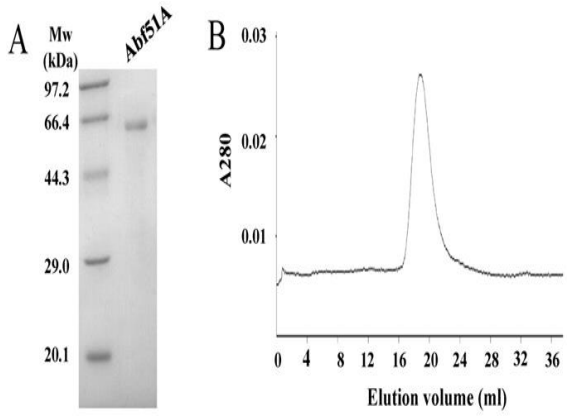

Figure 4: SDS-PAGE analysis (a) and gel filtration chromatography profile (b) of Abf51A.

5.4. Biochemical characterization of the recombinant enzyme

Rapid screening of Abf51A activity using different aryl-monosaccharides confirmed that Abf51A was only specific for *p*NP-Af(2770.83 U/mg) and *p*NP-β-Xyl (2.32 U/mg), but not for other aryl-glycosides including*p*NP-Ace,*p*NP-α-Xyl, *p*NP-C and *p*NP-Glu. Further testing on polymeric substrates revealed that Abf51A could release trace amount of reducing sugars from a variety of xylan polymersincluding OSX, CCX and SCX with specific activity of 0.006, 0.003 and0.001 U/mg,respectively.Besides, no activities were observed forlocust bean gum, soluble starch, Avicel and CMC. These results indicated that the recombinant Abf51A was an exo-type arabinanase. The Michaelis-Menten constant K_m and V_{max} values for *p*NP-Af under standard assay conditions were 0.52 ± 0.05 mM and 2981.00 ± 79.65 μmol/min/mg, respectively.

Analysis of enzymatic activity revealed that optimal temperature and pH for Abf51A were 80°C and 5.5, respectively. Abf51A retained more than 55% of its activity at 60-85°C, and maintained over 60% of the activity at pH 4.5-6.5, while decreased rapidly of more than 60% of itsactivity at pH 7.0-8.5, suggesting its thermophilic and acidic activity in solution (Figure.5a). Moreover, Abf51A was incubated at 75, 80 and 85°C in the absence of substrate for thermostability determination **(Figure.5b)**. Abf51A withstood long-term incubation at 75°C, retaining 63.0% of its original activity after 12 h at this temperature. Even at 80°C, 56.9% of the residual activity was left after 12 h, while 57.0% of the enzyme's activity dropped sharply at 85°C after 30 min.

Moreover, several properties of Abf51A were compared with some other thermophilic AFases from bacteria and fungi as shown in Table 1.The activity of thermostable AFases was maximal at pH 5.0-7.0. Abf51A was evidently distinguished from other AFases with much higher V_{max} value for *p*NP-Af. Besides, together with Abf51A, some AFases also had scant activity towards *p*NP-β-Xyl and xylan polymers.

5.5. Synergy with endo-β-1,4-xylanase

The applications of AFase in combination with endo-β-1,4-xylanase was studied in the hydrolysis of arabinose-rich xylans(Figure.6).Under the tested conditions (80°C, pH 6.0 for 24 h), both Abf51 and Xyn10Agave optimal activities, andremained more than 55% of their original activities after prolonged incubation. The pronounced release of reducing sugars from all tested xylans were achieved in the presence of Xyn10A compared with the separate use of Abf51A, but maximal substrate hydrolysis was attained in the combination of the two enzymes (Figure. 6a).During the hydrolytic process, a large amount of xylose was produced by Xyn10Aalone since xylan was mainly consisted of β-1,4-linked D-xylopyranosyl backbone. While much more xylose was released by the simulta-

neous action of Xyn10A and Abf51A, resulting in the highest 2.46-fold increase over the action of Xyn10A alone towards OSX (Figure. 6b). At the same time, Abf51A individually liberatedthe highest number of arabinose from OSX, but led to the biggest enhancement of arabinose production by 2.25 fold together with Xyn10A on CCX (Figure. 6c).The most significant synergy between Xyn10A and Abf51A was obtained with OSX as substrate compared with that of CCX and SCX**(Figure. 6d)**.

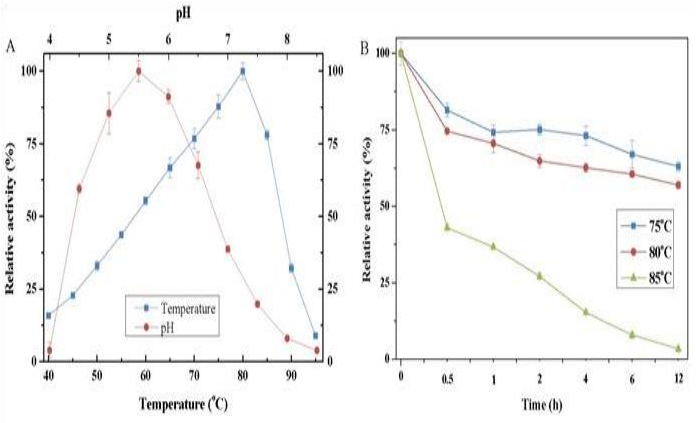

Figure 5: Effects of temperature and pH on the activity and stability of the recombinant Abf51A.(a) Temperature and pH optimum. (b) Temperature stability. Optimum temperature was measured at temperatures ranging from 40 to 95°C in phosphate-citrate buffer (pH 6.5). Similarly, optimum pH was determined in phosphate-citrate buffers (pH 4.0-8.5) at 75°C. For investigating thermostability, purified proteins were respectively incubated at 75, 80 and 85°C for different time periods in the absence of substrate. The residual activity was assayed under optimal conditions with pNP-Af as substrate. The highest activity was defined as 100% in triplicate.

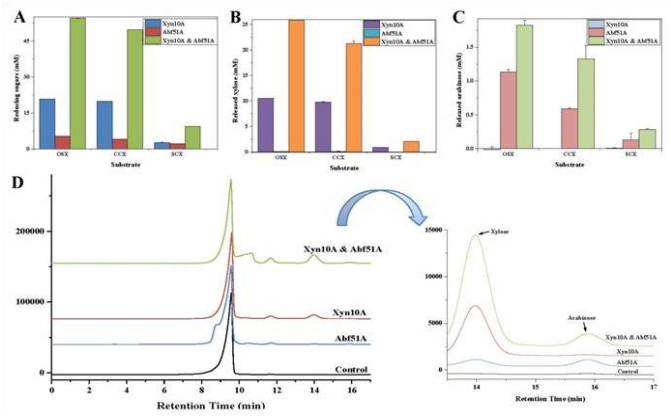

Figure 6: Synergistic hydrolysis of arabinose-rich xylans.(a) Reducing sugars assay. (b) Released xylose from xylans. (c) Released arabinose from xylans. (d) HPLC analysis of the hydrolytic products of OSX. The reactions were conducted in 1 mL mixture (pH 6.0) containing 25 µg purified protein (Abf51A and Xyn10A, either alone or in combination) and 1% (w/v) xylans (OSX, CCX and SCX) at 80°C for 24 h. The results represented the average of three experimental repeats.

Microorganism	Mw [a] (kDa)	Topt (°C)	pHopt	V_{max} [b] (µmol/min/mg)	K_m [b] (mM)	Spe act [c] (U/mg)	Xylan polymer attacked	References
C. lactoaceticus	57 (340)	80	5.5	2981.00	0.52	2.32	OSX, CCX, SCX	This study
Anoxybacilluskestanbolensis	58 (230)	65	5.5	1019.00	0.14	0	SBA, AX, OSX	Canakci et al. 2008
Geobacilluscaldoxylolyticus TK4	59 (236)	75-80	6.0	588.20	0.17	151.50 [d]	NA	Canakci et al. 2007
Thermobacillusxylanilyticus	56 (NA)	75	5.6-6.2	555.00	0.50	NA	AX, LWX, OSX	Debeche et al. 2000
Paenibacillus sp. DG-22	57 (222)	60	6.0	306.10	3.50	0.02	NA	Lee and Lee 2014
Ruminococcusalbus 7	56 (NA)	50	6.0	270.30	1.05	< 0.01	BeeWX, WFA	Yang et al. 2016
Caldicellulosiruptor saccharolyticus	58 (460)	80	5.5	186.00	1.29	0	NA	Lim et al. 2010
Thermotoga thermarum DSM5069	55 (NA)	95	5.0	162.97	0.19	NA	NA	Xie et al. 2016
Paenibacillus sp. HanTHS1	57 (NA)	75	5.0	NA	0.31	1.70	BirWX, BeeWX	Bouraoui et al. 2016
Thermotoga maritima MSB8	55 (332)	90	7.0	NA	0.42	2.02 [d]	NA	Miyazaki 2005
Clostridium sterc-orarium	52 (195)	70	5.0	NA	NA	< 0.01	OSX	Schwarz et al. 1995
Geobacillus sp. KCTC 3012	65 (NA)	60	6.0	NA	NA	NA	OSX	Arti et al. 2012

***Abbreviations:** Mw, molecular weight; Topt, optimum temperature; pHopt, optimum pH; Spe act, Specific activity; NA, not available; OSX, oat spelt xylan; CCX, corncob xylan; SCX, sugarcane xylan; SBA, sugar beet arabinan; AX, arabinoxylan; LWX, larchwoodxylan; BeeWX, beechwoodxylan; WFA, wheat flour arabinoxylan; BirWX, birchwoodxylan.

a Mw, molecular weight presented as Mw by SDS-PAGE (Mw by gel filtration/ mass spectrometry)

b Value for pNP-Af.

6. Discussion

Abf51A was the only AFase identified from the genome of *C. lactoaceticus*, and showed high sequence identity with family 51 AFases from*Caldicellulosiruptor* sp. Correspondingly, the model built for Abf51A displayed a typical GH51 two-domain structure, organized with a $(\beta/\alpha)_8$ folded catalytic domain and a β-sandwich folded domain at the C-terminal. The conserved Glu173 and Glu292 were proposed to be the acid-base and nucleophile catalytic residue, respectively. Additionally, like Abf51A, someGH51 AFases also had hexameric structure in solution [15-17]. All these results were in agreement with crystal structures of GH51 AFase from *G. stearothermophilus* T-6 [PDB: 1PZ3] [18], *C. thermocellum* [PDB: 2C7F] [19], *T. maritima* MSB8

[PDB: 4ATW] [20], *T. petrophila*RKU-1[PDB: 3S2C] [21] and *T. xylanilyticus* [PDB: 2VRK] [22]. So far, GH51 Tm-AFase from *T.thermarum*DSM5069 was the most thermophilic AFase purified and characterized with optimal activity up to 95°C [23]. Recombinant Abf51A also showed excellent thermostability with optimal activity at 80°C, responding to the characterization of other thermophilic AFases [24-26]. Substrate specificity study revealed that Abf51A had narrow substrate range and mainly cleaved the glycosidic bond linked in *p*NP-Af. Abf51A was also active on*p*NP-β-Xyl, but in contrast to GH 43 AFases,this activity was rather low. Similar substrate preference had also been described with some GH51 enzymes [27-35]. The explanation for this flexibility depended on the stereochemical relatedness of α-L-arabinosyl and β-D-xylopyranosyl moieties [36]. However, no biofunctionality had been claimed on this basis [37]. To date, the majority of characterized GH51 members displayed only AFase activity, while only a few were confirmed to be multi-functional. For example, the THSAbf from *Paenibacillus* sp. displayed both AFase and endo-xylanase activity, and was active on 4-*O*-methylglucuronoxylans from birch and beechwood [38]. In all cases, it was worth noticing that Abf51A had significant higher activity with *p*NP-Af as substrate although the affinity was not outstanding. As for Abf51A, it also displayed expected activity towards AX and GAXs including CCX and SCX. AX, appearing as the neutral or slightly acidic polymer, consists of β-1,4-linked D-xylopyranosyl residues to which α-L-arabinofuranosyl units are linked at C(O)-2 and/or C(O)-3 position. Moreover, phenolic acids such as ferulic and coumaric acids have been found to be esterified to the C(O)-5 position of arabinose [39]. According to statistics, AX has been extracted from a variety of the main commercial cereals including wheat, barley, oat, rye, rice and corn, sorghum as well as some grasses [40,41]. Generally, AX from rye, wheat and sorghum on average has a relatively high degree of substituted xylose residues, whereas both oats and barley contain higher levels of β-glucan than AX [39]. For example, the proportion of AX was once isolated from barley endosperm, accounting for just 25% of the total polysaccharides in cell walls [40]. However, the degree of substitution referring to the arabinose moieties on the xylose backbone varies significantly according to the cereal species.OSX, as a popular representative of AXinvestigated in many literatures, contains α-L-arabinofuranosyl residue at the *O*-3 position of the linear xylan backbone at every eight to ten xylose molecules [41]. In earlier reports, CCX revealed the existence of two structurally different forms: a low-branched water-insoluble GAX with about 95% of the backbone unattached, and a water-soluble heteroxylan with over 15% of the backbone substituted [41,42]. While xylan extracted from corn fiber contains about 52% xylose, along with 34% arabinose [43]. In contrast, heteroxylan isolated from sugarcane bagasse is typical acetylated GAX, with a glycosyl substitution molar ratio between the xylose units, *O*-3 linked terminal arabinose units and the *O*-2 linked glucuronic units of 10:0.5:0.1 [44]. In view of these facts, thedetectable activity of Abf51A with polymeric xylan was due to thehydrolysis of the C(O)2- and/or C(O)3-linkages between α-L-arabinofuranosyl substituents and backbone residues of AX and these GAXs. From a larger perspective, the available fermentable sugars are essential for efficient conversion of lignocellulosic biomass to ethanol and other valuable bio-based products. Hence the importance of side-chain-degrading enzymes like AFase comes from the fact that these substituentsextensively exist in the cross-linking structure of hemicellulose within plant cell wall. Besides, the enzymatic products from these heteroxylan, for instance,arabinoxylan-oligosaccharides, have been testified to be physiologically functional polysaccharides that exert prebiotic activities. Synergy of hydrolytic enzymes for complete degradation of arabinose-rich hemicelluloses to basic units has been successfully recorded in previous studies, which is a feasible way to improve sugar yield at low cost. For example, novel synergistic associations among AFase, endo-β-1,4-xylanase and endo-1,4-β-mannanase had already been established to degrade the hemicelluloses of alkaline pre-treated sugarcane bagasse. Respond to the results of this study, significant synergistic effects between AFase and other xylanolytic degrading enzymes had also been widely acknowledged. In these cases, efficient processing of xylan could be achieved by the predominant de-polymerization of the main bone by endo-β-1,4-xylanase to assimilate oligosaccharides, together with branch removal by the de-branching xylanolytic system. Cooperation *C. stercorarium*ArfB with endo-xylanase on OSX yielded products of arabinose, xylose, xylobiose and xylotriose after prolonged incubation. *Paenibacillus* sp. JDR-2 assimilated methylglucuronoarabinoxylan from sugarcane by the synergy between AFase and GH10/GH67 xylan utilization enzymes. Hence, when Abf51A was incubated with endo-β-1,4-xylanase Xyn10A to simultaneously degrade AX and GAXs, more reducing sugars were released.However, the difference in the initial reaction rates and products for the tested substratesmight be attributed tothe distribution diversity of substitutesand the linkage preference of AFase. In conclusion, theGH51 AFasesAbf51A from extremely thermophilic bacterium *C. lactoaceticus*exhibited remarkable activity onboth *p*NP-Af and *p*NP-β-D-Xyl. Abf51A showed optimal activity at 80°C and pH 5.5 with favorable thermostability. Moreover, Abf51A has the accessory function of L-arabinofuranosyl substituents removal and acted synergistically with endo-β-1,4-xylanase in AX and GAX degradation. The excellent thermostability, hydrolytic properties and synergism with endo-β-1,4-xylanase suggested that Abf51Awas an optimum enzyme for pentosesproduction from arabinose-rich xylans.

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