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Cloning, Expression, and Characterization of a Thermostable GH51 α-L-Arabinofuranosidase from *Paenibacillus* sp. DG-22

Sun Hwa Lee and Yong-Eok Lee*

Department of Biotechnology, Dongguk University, Gyeongju 780-714, Republic of Korea

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**Corresponding author*
Phone: +82-54-770-2226;
Fax: +82-54-770-2287;
E-mail: yelee@dongguk.ac.kr

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The gene encoding α-L-arabinofuranosidase (AFase) from *Paenibacillus* sp. DG-22 was cloned, sequenced, and expressed in *Escherichia coli*. The AFase gene (*abfA*) comprises a 1,509 bp open reading frame encoding 502 amino acids with a molecular mass of 56,520 daltons. The deduced amino acid sequence of the gene shows that AbfA is an enzyme consisting of only a catalytic domain, and that the enzyme has significant similarity to AFases classified into the family 51 of the glycosyl hydrolases. *abfA* was subcloned into the pQE60 expression vector to fuse it with a six-histidine tag and the recombinant AFase (rAbfA) was purified to homogeneity. The specific activity of the recombinant enzyme was 96.7 U/mg protein. Determination of the apparent molecular mass by gel-filtration chromatography indicated that AbfA has a tetrameric structure. The optimal pH and temperature of the enzyme were 6.0 and 60°C, respectively. The enzyme activity was completely inhibited by 1 mM $HgCl_2$. rAbfA was active only towards *p*-nitrophephenyl α-L-arabinofuranoside and exhibited K_m and V_{max} values of 3.5 mM and 306.1 U/mg, respectively. rAbfA showed a synergistic effect in combination with endoxylanase on the degradation of oat spelt xylan and wheat arabinoxylan.

Keywords: α-L-Arabinofuranosidase, *Paenibacillus* sp. DG-22, gene cloning and expression

Introduction

Xylan is a major component of hemicellulose and is the second most abundant polysaccharide in plant cell walls [36]. It consists of a linear backbone of α-1,4-linked D-xylopyranosyl units, which are substituted with different side chains with L-arabinose, acetyl, and glucuronic residues. Therefore, complete degradation of xylan requires the concerted action of several different enzymes such as endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, and acetyl xylan esterase [5, 34]. α-L-Arabinofuranosidases (AFases; E.C. 3.2.1.55) catalyze the hydrolysis of α-linked L-arabinofuranosyl residues at the nonreducing ends of arabinose-containing hemicelluloses such as arabinoxylans, arabinans, and arabinogalactans [14]. The presence of L-arabinofuranoside side chains restricts the action of endo-acting xylanases. Therefore, the AFases are the most important accessory enzymes involved in synergy with other hemicellulases for the complete hydrolysis of hemicelluloses. These enzymes have been purified from several bacteria, fungi and plants [25, 30].

Based on amino acid sequence similarities and hydrophobic cluster analysis, AFases have been classified into five glycosyl hydrolase (GH) families; namely, GH3, GH43, GH51, GH54, and GH62 [12]. Among the various AFases, GH51 family AFases have been well studied at the structural level. To date, five crystal structures of GH51 AFases have been published. These are AFases from *Geobacillus stearothermophilus* T-6 [13], *Clostridium thermocellum* [35], *Thermobacillus xylanilyticus* [26], *Thermotoga petrophila* RKU-1 [33], and *Thermotoga maritima* MSB8 [3]. Analysis of these structures has revealed that GH51 AFases are composed of a catalytic domain characterized by a $(\beta/\alpha)_8$ barrel with two conserved glutamate residues in the catalytic site. AFases have also been classified into three types depending on their substrate specificities. Type-A AFases are not active toward polymers whereas type-B AFases are. The third type of AFases, arabinofuranohydrolases, are specific for arabinoxylans [30]. Bifunctional AFases that have both β-xylosidase and α-L-arabinofuranosidase

activities have also been reported [7, 24, 37].

α-L-Arabinofuranosidases have potential applications in several biotechnological processes, such as improvement of animal feedstock digestibility and wine flavors, juice clarification, and delignification of pulp [25, 30]. In this respect, thermostable AFases have considerable industrial interest.

Paenibacillus sp. DG-22, a moderately thermophilic bacterium, grows actively on xylan as the sole carbon source and does not have cellulase activity [20]. We previously reported the purification and characterization of endoxylanases [21] and cloning of the xylanase A gene [19] and β-xylosidase gene [18] from this bacterium. To understand the entire function of the xylanolytic system of *Paenibacillus* sp. DG-22, investigations of hemicellulases other than xylanases and their genes are necessary. In this study, the gene encoding α-L-arabinofuranosidase from *Paenibacillus* sp. DG-22 was cloned and expressed in *Escherichia coli*. The recombinant enzyme was purified and its biochemical properties were characterized.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Paenibacillus sp. DG-22 was grown as previously described [21] and was used as the source of genomic DNA. *E. coli* DH5α and the plasmid pUC19 were used for genomic library construction. Transformants were grown in LB medium, consisting of 1% peptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.0), supplemented with 50 μg/ml of ampicillin. Plasmid pQE60 (Qiagen, Germany) was used as an expression vector to construct a recombinant enzyme containing a six-histidine tag at its carboxy terminus, and *E. coli* M15 (pREP4) (Qiagen) was used as an expression host. The recombinants were grown in LB medium supplemented with ampicillin (50 μg/ml) and kanamycin (25 μg/ml).

Genomic Library Construction and Screening of AFase-Positive Clones

The chromosomal DNA of *Paenibacillus* sp. DG-22 was isolated by the method of Marmur [22]. The genomic DNA was partially digested with the restriction enzyme *Sau*3AI and the fragments of 3–5 kb fractions were excised from the agarose gel and purified using a gel extraction kit (GeneAll, Korea). These DNA fragments were ligated into the dephosphorylated *Bam*HI site of pUC19 and transformed into *E. coli* DH5α to construct a genomic library. AFase-positive clones were screened using the fluorogenic substrate 4-methylumbelliferyl α-L-arabinofuranoside (αMUA) (Sigma, USA). Top agar (0.7% (w/v)) containing αMUA (10 μg/ml) was overlaid onto the agar plate and incubated at 60°C for 1 h. Positive clones were identified by observing fluorescence under UV light.

DNA Sequencing and Sequence Analysis

DNA sequencing was conducted at the Genotech DNA sequencing facility (Daejeon, Korea) by automated sequencing using the dideoxynucleotide chain termination method. The nucleotide sequence was analyzed using the National Center for Biotechnology Information (NCBI) Open Reading Frame (ORF) Finder tool. The signal peptide in the deduced amino acid sequence was predicted by the SignalP 4.0 server [28]. Homology searches in the GenBank database were carried out using the BLAST program [1]. Multiple sequence alignments were carried out with the Clustal Omega program [32].

PCR and Subcloning into Expression Vector

For expression and purification of the AFase from *Paenibacillus* sp. DG-22 (AbfA) in *E. coli*, the *abfA* coding region was amplified by PCR using *abfA* cloned in pUC19 as a template. Forward (5′-GAGGTGATCCATGGCAAGCGCGGCGA-3′, *Nco*I site underlined) and reverse (5'-CGCTTGCCTTAGATCTAAACCGAAGC-3', *Bgl*II site underlined) primers were designed and synthesized to contain *Nco*I and *Bgl*II recognition sites to allow in-frame cloning into the pQE60 expression vector. PCR was carried out in a MyCycler thermal cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 60 sec, annealing at 62°C for 60 sec, and extension at 72°C for 90 sec, and a final extension step at 72°C for 10 min. The PCR product was gel-purified, digested with *Nco*I and *Bgl*II, and ligated into the corresponding sites of pQE60. The resulting plasmid was designated pQE60-abfA, and used to transform *E. coli* M15(pREP4). pQE60-abfA encodes a fusion protein that consists of AbfA plus a C-terminal tag with six histidines.

Purification of the Recombinant α-L-Arabinofuranosidase

The recombinant enzyme was purified from *E. coli* M15(pREP4) harboring pQE60-abfA. The recombinant strain was grown in 500 ml of LB medium containing ampicillin (50 μg/ml) and kanamycin (25 μg/ml) at 37°C. After the optical density at 600 nm reached 0.6, the culture was induced with 1 mM IPTG for 4 h. The cells were harvested by centrifugation (4,000 ×*g* for 30 min at 4°C), and resuspended in 20 ml of lysis buffer (50 mM sodium phosphate, 0.3 M NaCl, and 10 mM imidazole, pH 8.0) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. The cell suspension was disrupted by sonication using a SONIFIER 450 (Branson, USA) on ice, and cell debris was removed by centrifugation (7,000 ×*g* for 30 min at 4°C). The cell extract was heat-treated at 55°C for 30 min in a water bath, and then chilled on ice. The denatured proteins were removed by centrifugation (7,000 ×*g* for 30 min at 4°C). The resulting supernatants were loaded onto a 5 ml Ni-NTA affinity column (Qiagen), washed with wash buffer (50 mM sodium phosphate, 0.3 M NaCl, and 100 mM imidazole, pH 8.0), and eluted with elution buffer (50 mM sodium phosphate, 0.3 M NaCl, and 250 mM imidazole,

pH 8.0). Active fractions were identified, combined, and desalted by dialysis against 50 mM sodium phosphate buffer (pH 6.0). The purified enzyme was used for analysis of enzymatic properties.

Enzyme Assay, SDS-PAGE, and Gel Filtration Chromatography

α-L-Arabinofuranosidase activity was measured by a spectrophotometric method with *p*-nitrophenyl α-L-arabinofuranoside (*p*NPA; Sigma) as the substrate. The reaction mixture, composed of 1 mM *p*NPA, 50 mM sodium phosphate (pH 6.0), and diluted enzyme in a 0.4 ml reaction volume, was incubated at 60°C for 10 min. The reaction was stopped by the addition of 0.8 ml of 1.0 M Na_2CO_3 and the *p*-nitrophenol released was measured as the absorbance at 410 nm. A standard curve was prepared using *p*-nitrophenol. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol in 1 min. Other *p*-nitrophenyl glycosides were also tested as substrates under the same conditions. Xylanase activity was determined by measuring the release of reducing sugars from xylan as previously described [19].

The protein content was determined by the Bradford method [6] with Protein Assay reagent (Bio-Rad) using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 15% running gel [17] and resolved proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma). The native molecular mass of AbfA produced by *E. coli* was determined using a Superdex 200 column with a fast-performance liquid chromatography (FPLC) apparatus (GE Healthcare). The calibration was performed with thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) as standards.

Biochemical Characterization of the Recombinant Enzyme

The effect of pH on the activity of the recombinant AbfA (rAbfA) was investigated at various pH values ranging from 4.0 to 9.0 at 60°C. The following buffers (each at 50 mM) were used: sodium acetate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 7.5), and Tris-HCl (pH 7.5 to 9.0). The pH stability was determined by pre-incubating the purified enzyme in the absence of substrate at pH ranging from 4.0 to 10.0 at 50°C for 2 h, and then the remaining activity was measured under standard conditions. The effect of temperature on the activity was estimated by incubating the purified enzyme at different temperatures in the range of 30°C to 75°C at pH 6.0. The thermostability of the purified enzyme was monitored by pre-incubating the enzyme in the absence of substrate at 60°C, 65°C, and 70°C. After various times, aliquots were withdrawn and the residual activity was measured under standard assay conditions.

The substrate specificity of the purified rAbfA was tested using various *p*-nitrophenyl glycosides (1 mM each; Sigma) and xylans (1% (w/v)). The effect of metal ions and some chemicals on recombinant enzyme activity was assessed in the presence of 1 and 10 mM test compounds under standard conditions. The enzymatic activities were expressed as a percentage of the activity obtained in the absence of compound. For determination of kinetic parameters, the purified enzyme was incubated with *p*NPA at various concentrations (ranging from 4 to 18 mM) under optimal conditions. The K_m and V_{max} values were calculated from a Lineweaver-Burk plot of the Michaelis-Menten equation.

Synergy with Xylanase

To investigate the synergistic effect of rAbfA in combination with xylanase, endoxylanase (XynA) from *Paenibacillus* sp. DG-22 was purified as previously described [19]. The reaction mixture (1 ml) contained 10 mg of oat spelt xylan (Sigma) or medium viscosity wheat arabinoxylan (Megazyme, Ireland), 50 mM sodium phosphate buffer (pH 6.0), and XynA (0.25 U/ml) and rAbfA (0.5 U/ml) either alone or in combination. After incubation at 50°C for 30 min, the mixtures were boiled for 10 min, and centrifuged at 20,000 ×*g* for 5 min to remove the unhydrolyzed xylan. Released reducing sugars in the supernatant were measured by the DNS method [23] as xylose equivalents.

Nucleotide Sequence Accession Number

The nucleotide sequence of *abfA* has been deposited in the GenBank database under the accession number KF373080.

Results and Discussion

Cloning and Sequence Analysis of the α-L-Arabinofuranosidase Gene

A genomic library of *Paenibacillus* sp. DG-22 was constructed using pUC19 as a cloning vector and AFase-positive clones were screened using the fluorogenic substrate αMUA. One positive clone was found and AFase activity was confirmed by the enzyme assay. A recombinant plasmid, designated pAC1, was isolated from this clone, and the entire region of the insert DNA was sequenced. The insert is about 4.5 kb in length, containing one complete open reading frame (ORF) of 1,509 bp. This ORF encodes a hypothetical protein of 502 amino acids with a calculated molecular mass of 56,520 dalton and a *p*I of 5.96. The ORF begins with an ATG and terminates with a TAA codon, and no signal peptide was predicted based on SignalP 4.0 analysis. Upstream of the coding region, we found putative -35 (TTGCTT) and -10 (TTTAAT) sequences, with 17 bp spacing. A putative ribosome binding sequence, 5′-AGGTGA-3′, was found 5 bp upstream of the potential ATG initiation codon. The TAA stop codon was followed by an inverted repeat that probably constitutes a transcription terminator.

The deduced amino acid sequence of AbfA was compared with the sequences in GenBank using the online BLAST program (BLASTp algorithm). The AbfA sequence showed that the enzyme consists of only a catalytic domain, and that it has significant similarity with GH family 51 AFases.

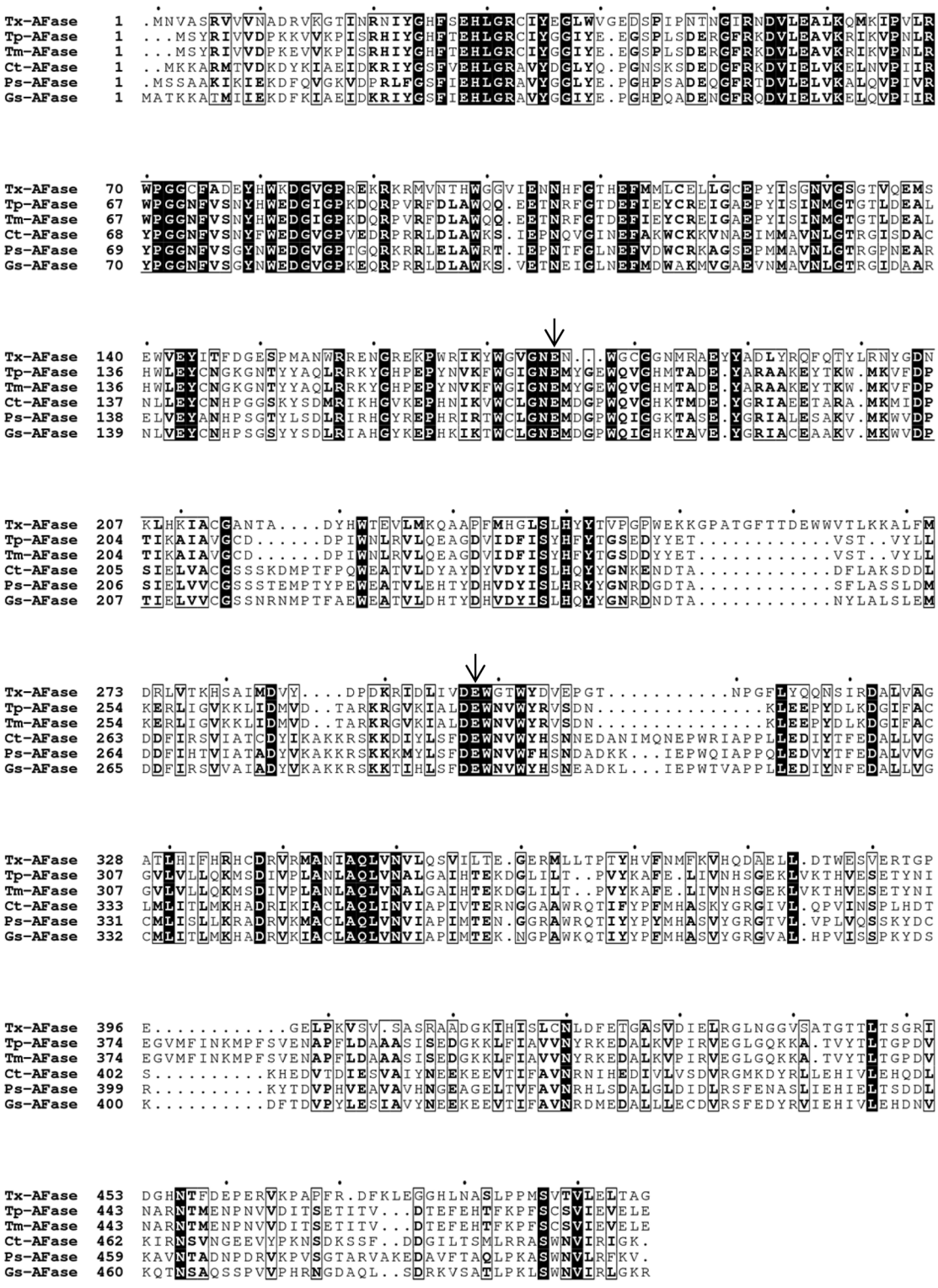

Fig. 1. Multiple amino acid sequence alignment of AbfA from *Paenibacillus* sp. DG-22 with other crystallized GH 51 AFases.
Identical and similar amino acid residues are enclosed in black boxes and open boxes, respectively. The two catalytic glutamate residues are marked above with arrows. The alignment includes the AFases from *Paenibacillus* sp. DG-22 (Ps-AFase), *T. xylanilyticus* (Tx-AFase, PDB code 2VRQ), *T. petrophila* (Tp-AFase, 3S2C), *T. maritima* (Tm-AFase, 4ATW), *C. thermocellum* (Ct-AFase, 2C7F), and *G. stearothermophilus* (Gs-AFase, 1PZ3). The figure was prepared using ESPript ver. 2.2 [11].

The highest identity was 75% with the AFase from *Paenibacillus* sp. JDR-2 (GenBank Accession No. YP_003013427). AbfA from *Paenibacillus* sp. DG-22 also has much sequence similarity with AFases from *Geobacillus stearothermophilus* T-6 (68% identity, ABM68633), *Clostridium thermocellum* ATCC 27405 (61% identity, YP_001038942), *Thermotoga petrophila* RKU-1 (34% identity, YP_001244227), *Thermotoga maritima* MSB8 (34% identity, NP_228093), and *Thermobacillus xylanilyticus* (27% identity, CAA76421).

To date, five crystal structures of family GH51 bacterial AFases from *G. stearothermophilus* T-6 [13], *C. thermocellum* ATCC27405 [35], *T. xylanilyticus* [26], *T. petrophila* RKU-1 [33], and *T. maritima* MSB8 [3] have been reported. Analysis of these structures has revealed that GH51 AFases are composed of a catalytic domain characterized by a $(\beta/\alpha)_8$ barrel, and two essential glutamate residues are required for catalytic function. The alignments of amino acid sequences of these AFases along with the AbfA from *Paenibacillus* sp. DG-22 are given in Fig. 1. The two catalytic glutamates are well conserved in all of the aligned sequences. Residues Glu175, identified as the acid/base, and Glu294, identified as the nucleophile, in *G. stearothermophilus* T-6 AbfA [13] are conserved as Glu174 and Glu293, respectively, in AbfA from *Paenibacillus* sp. DG-22 (Fig. 1).

Expression and Purification of the Recombinant AbfA

To facilitate the purification of recombinant AFase by affinity chromatography, AbfA was expressed as a fusion protein containing a six-histidine tag at its C-terminus. The coding region of *abfA* was amplified by PCR and cloned into the expression vector pQE60 in-frame, resulting in the plasmid pQE60-abfA. The histidine-tagged recombinant AbfA (rAbfA) was purified in two steps: heat treatment and immobilized metal affinity chromatography (IMAC). Purified rAbfA showed a specific activity of 96.7 U/mg to *p*NPA. A summary of the purification procedure is shown in Table 1. Heat treatment of cell extracts at 55°C for 30 min increased the specific activity 3.6-fold, with a recovery yield of 91.6%. Final purification was performed by IMAC with an Ni-NTA column (Qiagen). The rAbfA was purified about 7.4-fold in specific activity, with a recovery yield of 59.1%. The purified enzyme showed a single band with a molecular mass of ~57 kDa on the SDS-PAGE gel (Fig. 2). The results of SDS-PAGE agreed with the size (57.3 kDa) calculated from the deduced amino acid sequence including the six-histidine tag. The molecular mass of the native rAbfA was 221.5 kDa as determined by gel-filtration chromatography (data not shown). Therefore, the quaternary structure of rAbfA was concluded to be a tetramer, as is the case with AFases from *Bacillus pumilus* [9] and *G. stearothermophilus* [10].

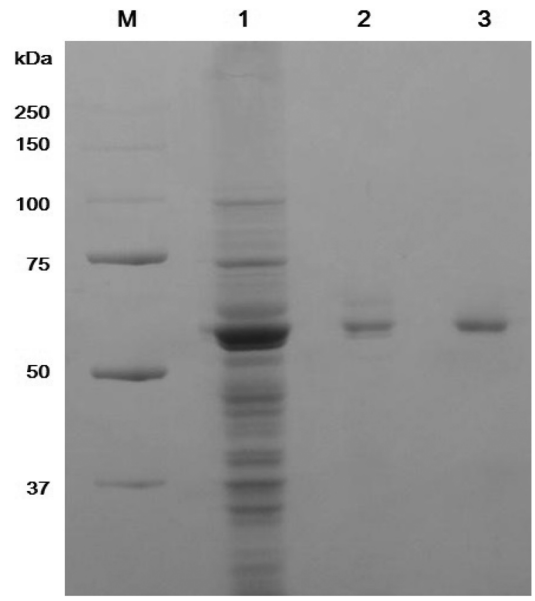

Fig. 2. SDS-PAGE analysis of the purification of rAbfA expressed in *E. coli*.
Lane M, molecular weight marker; lane 1, cell extract of the induced transformant harboring pQE60-AbfA; lane 2, proteins after heat treatment; lane 3, purified rAbfA after Ni-NTA affinity chromatography.

Effects of pH and Temperature on Enzyme Activity and Stability

The biochemical properties of purified rAbfA were investigated. The activity of rAbfA was determined in three different buffers covering the range between pH 4 and 9 at 60°C using *p*NPA as the substrate. The purified

Table 1. Purification of rAbfA from *E. coli* M15 (pREP4) harboring pQE60-AbfA.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	137	1,760	12.8	1.0	100
Heat treatment	35	1,613	46.1	3.6	91.6
IMAC	11	1,040	94.5	7.4	59.1

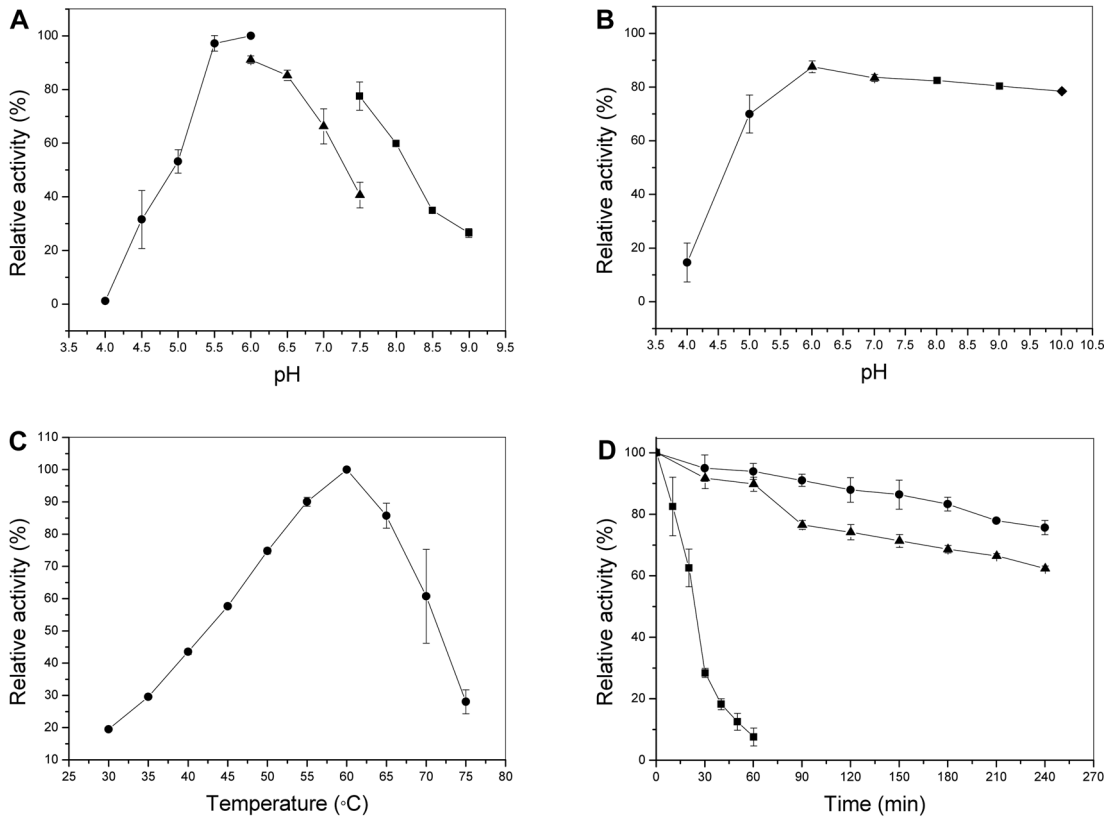

Fig. 3. Effects of pH and temperature on the activity and stability of purified rAbfA.

The activities at the optimal pH and the optimal temperature were defined as 100%. (**A**) Effect of pH on rAbfA activity. (**B**) The pH stability of the enzyme. The residual activity was determined after incubating the enzyme at 50°C for 2 h in buffer at pH 4.0–10.0. The initial activity was defined as 100%. Buffers used: sodium acetate (●), sodium phosphate (▲), Tris-HCl (■), glycine-NaOH (◆). (**C**) Effect of temperature on rAbfA activity. (**D**) The thermostability of purified rAFase at pH 6.0 in the absence of substrate. Residual activity was monitored at various times after incubation at 60°C (●), 65°C (▲), and 70°C (■). The initial activity was defined as 100%.

enzyme showed a pH optimum of 6.0 and retained 30% of its activity at pH 4.5 and 9.0 (Fig. 3A). This optimal pH is typical for AFases from bacteria, whereas most fungal AFases have pH optima of less than 5.0 [30]. The purified enzyme was fairly stable from pH 6.0 to 9.0, as more than 85% of its activity was maintained when the enzyme was treated at 50°C for 2 h (Fig. 3B). The activity of the recombinant enzyme was also measured at temperatures between 30°C and 75°C. For 10 min reactions, the optimal temperature was 60°C and the enzyme still retained 86% activity at 65°C (Fig. 3C). To examine the thermostability of rAbfA, the purified enzyme was incubated at 60°C, 65°C, and 70°C without substrate for up to 4 h, and the residual activities were measured. In the absence of substrate, rAbfA retained 63% of its initial activity after 4 h of pre-incubation at 65°C, but the activity decreased rapidly at 70°C. The half-life of rAbfA was about 25 min at 70°C (Fig. 3D).

Effects of Metals and Other Chemicals

The activity of rAbfA was measured under standard assay conditions in the presence of several metal ions and chemicals, including EDTA, DTT, β-mercaptoethanol, and SDS (Table 2). The results indicate that rAbfA activity is completely inhibited by Hg^{2+} and significantly inhibited by Ag^{+}, Co^{2+}, Cu^{2+}, and Zn^{2+} cations even at a concentration of 1 mM. The enzyme has no apparent requirement for metal ions. Inhibition by Hg^{2+} seems to be a general property of AFases [7, 8, 10, 27], indicating the existence of thiol groups in the catalytic site of the enzyme [4]. rAbfA activity was moderately inhibited by Ca^{2+}, Fe^{2+}, K^{+}, and Mg^{2+}, and no effect on activity was detected with Mn^{2+}, Na^{+}, and Rb^{+}.

Table 2. Effects of metal ions and selected chemicals on rAbfA activity.

Compound[a]	Relative activity[b] (%)	
	1 mM	10 mM
Control	100	100
AgCl	0.4	0.1
$CaCl_2$	16.7	0.5
$CoCl_2$	2.2	1.4
$CuCl_2$	1.5	1.2
$FeCl_2$	55.8	4.9
$HgCl_2$	0	0
KCl	34.2	5.3
$MgCl_2$	43.9	29.8
$MnCl_2$	105.0	58.3
NaCl	94.9	91.3
RbCl	94.0	69.6
$ZnCl_2$	3.9	0.3
EDTA	106.3	105.9
β-Mercaptoethanol	165.4	123.3
DTT	180.4	150.2
SDS	28.9	4.2

[a]All reagents were dissolved in deionized water.

[b]The activity assayed in the absence of compounds was taken as 100%. Values presented are the means of three independent experiments.

The addition of the chelating agent EDTA did not inhibit AFase activity, suggesting that no metals are needed for enzymatic activity. The reducing agents β-mercaptoethanol and DTT increased the relative activity by 165% and 180%, respectively, at a concentration of 1 mM (Table 2). This result indicates the possible presence of a thiol group of cysteine in the catalytic site [16], as these reducing reagents could prevent the oxidation of sulfhydryl groups [15]. Enzymatic activity was markedly inhibited in the presence of 10 mM SDS.

Substrate Specificity and Kinetic Analysis

The substrate specificity of rAbfA was determined by measuring its activity with various para- and ortho-nitrophenyl glycosides and several xylans as substrates (Table 3). rAbfA was highly active towards *p*-nitrophenyl α-L-arabinofuranoside, but showed only marginal activity towards *p*-nitrophenyl β-D-xylopyranoside. No detectable release of reducing sugars could be measured when rAbfA was incubated with polymeric xylans as substrates. These results indicate that rAbfA belongs to the type-A AFases, which are not active towards polymers [30]. Although most AFases usually exhibit a narrow range of substrate specificity, several AFases have been reported to have both β-xylosidase and α-L-arabinosidase activities [7, 24, 37].

Table 3. Substrate specificity of rAbfA for different nitrophenyl-glycosides and xylans.

Substrate	Specific activity (U/mg)[a]
p-Nitrophenyl α-L-arabinofuranoside	58.6 ± 6.6
p-Nitrophenyl α-L-arabinopyranoside	ND[b]
p-Nitrophenyl β-L-arabinopyranoside	ND
p-Nitrophenyl α-D-glucopyranoside	ND
p-Nitrophenyl β-D-glucopyranoside	ND
p-Nitrophenyl β-D-mannopyranoside	ND
p-Nitrophenyl β-D-xylopyranoside	0.018 ± 0.001
p-Nitrophenyl acetate	ND
o-Nitrophenyl β-D-galactopyranoside	ND
Beechwood xylan	ND
Birchwood xylan	ND
Oat spelt xylan	ND
Wheat arabinoxylan	ND

[a]Values are the means ± SD (n = 3).

[b]ND, not detected.

Table 4. Synergistic action of endoxylanase (XynA) and α-L-arabinofuranosidase (rAbfA) on oat spelt xylan and wheat arabinoxylan.

Enzyme(s)	Liberated reducing sugar (mg/ml)[a]	Degree of synergy[b]
Oat spelt xylan		
XynA	0.50 ± 0.01	
rAbfA	0	
XynA + rAbfA	0.68 ± 0.02	1.36
Wheat arabinoxylan		
XynA	0.33 ± 0.01	
rAbfA	0	
XynA + rAbfA	0.49 ± 0.01	1.48

[a]Reducing sugar was measured using the DNS method [23].

[b]These values were calculated based on the ratio between synergistic activity (XynA + rAbfA) and the sum of the activities of each enzyme separately [29].

The kinetic parameters of rAbfA were investigated using *p*NPA as a substrate. The purified enzyme exhibited typical Michaelis-Menten kinetics, with K_m and V_{max} values of 3.50 ± 0.16 mM and 306.11 ± 6.06 U/mg protein, respectively.

Synergy with Xylanase

To determine the synergistic effects between xylanase and AFase, we mixed rAbfA with endoxylanase (XynA) from *Paenibacillus* sp. DG-22 [19] and then compared the amount of reducing sugars released from arabinose-containing xylan substrates (Table 4). As expected, rAbfA exhibited mild but significant synergistic effects with endoxylanase on the degradation of oat spelt xylan and wheat arabinoxylan, with a 1.36- and 1.48-fold increase in the amount of reducing sugar released, respectively. This is probably due to the removal of arabinose residues from xylans by rAbfA, which results in elimination of steric hindrance and exposure of additional sites at which endoxylanase can act [31]. Synergistic interactions between AFases and xylanases in the hydrolysis of xylan have also been previously reported [2, 29, 31].

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