J. Microbiol. Biotechnol. (2004), **14**(3), 474–482

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of the *arf*A Gene from *Bacillus stearothermophilus* No. 236 and Its Protein Product, α-L-Arabinofuranosidase

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Received: May 19, 2003
Accepted: November 3, 2003

Abstract The α-L-arabinofuranosidase (Arfase) gene of *Bacillus stearothermophilus* No. 236 was cloned and sequenced. The ORF of the gene, designated *arfA*, encoded a 507-residue polypeptide with calculated molecular mass of 57 kDa. The Arfase produced by a recombinant *Escherichia coli* strain containing the *arfA* gene was purified to apparent homogeneity and characterized. The molecular mass of the Arfase determined by SDS-PAGE was 60 kDa. However, according to gel filtration, it was estimated to be approximately 190 kDa. These results indicated that the functional form of the Arfase is trimeric. The optimal pH and temperature for the enzyme activity were pH 6.5 and 55°C, respectively. The half-life of the enzyme at 60°C was about 6 h. Kinetic experiments at 45°C with pNPAf (*p*-nitrophenyl α-L-arabinofuranoside) as a substrate gave the K_m and V_{max} values of 1.19 mM and 26.1 U/mg, respectively. When the enzyme was combined with *Bacillus stearothermophilus* No. 236 endoxylanase and β-xylosidase, it hydrolyzed arabinoxylan into L-arabinose and xylose more efficiently than Arfase alone. This synergistic effect suggested that the complete hydrolysis of xylan with large amounts of arabinose side chains required Arfase as well as endoxylanase and β-xylosidase.

Key words: α-L-arabinofuranosidase, *arfA*, *Bacillus stearothermophilus* No. 236, synergistic effect, xylan hydrolysis

Xylans, next to cellulose, are the most abundant polysaccharides on earth, and they are included within plant cell wall heteropolysaccharides referred to as hemicelluloses. It has been estimated that about 10^{10} metric tons of xylans are recycled per year, mainly through the action of microbes. They consist of a β-1,4-linked xylopyranose backbone, to which are often attached side groups of arabinose, (*O*-methyl-) glucuronic acid, ferulic or *p*-coumaric acid, and/or acetate, depending on the plant sources [1, 2, 19, 22]. The common substituents found on the backbone of xylan are acetyl, arabinosyl, and glucuronysyl residues [21]. These side chains in xylan have been demonstrated to restrict the enzymatic hydrolysis of the backbone by the major xylanolytic enzymes including endoxylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) [3]. Thus, complete digestion of xylan requires not only the major xylanolytic enzymes but also the side chain-removing enzymes such as α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.1), and acetylxylan esterase (EC 3.1.1.72) [23]. Synergism between the xylanases and α-L-arabinofuranosidase was described for the enzyme system of *Talaromyces emersonii*. The xylanases II and III from *T. emersonii* were unable to hydrolyze wheat straw xylan (arabinoxylan), unless the polymer had been previously treated with an α-L-arabinofuranosidase of the same organism [20]. Also, in our labarotary, similar synergistic effects were observed among the xylanase, β-xylosidase, and α-L-arabinofuranosidase from *Bacillus stearothermophilus* No. 236 [17].

Along with the efforts to understand the biochemistry of xylan degradation on the molecular level and to establish an efficient xylanolytic process, we have studied the xylan-hydrolyzing system of *B. stearothermophilus* No. 236. So far, the genes responsible for endoxylanase, β-xylosidase, α-glucuronidase, and acetylxylan esterase have been cloned and their nucleotide sequences determined. All of their protein products were also purified and characterized biochemically [4, 10, 15].

In this paper, we report the cloning and sequencing of the α-L-arabinofuranosidase gene (*arfA*) of *B. stearothermophilus* No. 236. Purification and characterization of the enzyme produced by the recombinant *E. coli* strain carrying the *arfA* gene are also described.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

B. stearothermophilus No. 236 was grown at 45°C with shaking (200 rpm) in a basal medium (10.6 g K_2HPO_4, 6.1 g $NaH_2PO_4 \cdot 2H_2O$, 2.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, and 3.5 g yeast extract per liter) containing 0.5% arabinose as a carbon source [16]. *E. coli* XL1Blue was used as the host for the recombinant plasmids used in this work. The plasmids pUC118 and pBR322 were used in the cloning, sequencing, and expression experiments. The recombinant *E. coli* strain was grown at 37°C in a Luria-Bertani (LB) medium containing ampicillin (100 μg/ml). The reagents, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), were added to the medium when necessary to the concentrations of 200 and 100 μg/ml, respectively.

Chemicals

Birchwood xylan, oat spelt xylan, *p*-nitrophenol-β-D-xylopyranoside (pNPX), and *p*-nitrophenyl-α-L-arabinofuranoside (pNPAf) were purchased from Sigma (St. Louis, MO, U.S.A.). Arabinoxylan was obtained from Megazyme (Wicklow, Ireland). DAEA-Sepharose CL-6B and Sephacryl S-300 were purchased from Amersham Bioscience (Piscataway, NJ, U.S.A.). Other chemicals used were analytical grade reagents.

Cloning of the *arfA* Gene and DNA Techniques

Standard procedures were used in the isolation of plasmid and chromosomal DNAs, transformation, restriction endonuclease digestion, DNA ligation, and gel electrophoresis. To obtain the ArfA encoding gene, the *Bacillus stearothermophilus* No. 236 genomic DNA was digested with *Eco*RI. DNA fragments of 3-9 kb generated from the digestion reaction were ligated with the *Eco*RI-digested and dephosphorylated pBR322 DNA, and then transformed into the *E. coli* XL1Blue cells. Transformed cells were spread on LB agar plates supplemented with ampicillin and pNPAf, a chromogenic synthetic substrate, and incubated for 18 h at 37°C. The recombinant *E. coli* cells containing the *arfA* gene were selected by their ability to produce yellow pigment around the colonies on the selective medium as described above.

Biochemical Assays

The Arfase activity was measured by the spectrophotometric method with pNPAf as a substrate. The assay mixture contained 50 μl of the substrate solution (2 mM pNPAf in 50 mM sodium phosphate buffer, pH 6.5), and 50 μl of the appropriately diluted enzyme solution. After incubation at 45°C for 20 min, the reaction was terminated by adding 100 μl of 1 M Na_2CO_3. The liberated *p*-nitrophenol in the reaction mixture was measured by spectrophotometry at 405 nm. One unit of Arfase activity was defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol per min under the assay conditions described above. β-Xylosidase assay was based on the measurement of *p*-nitrophenol released from the substrate, *p*-nitrophenol-β-D-xylopyranoside (pNPX) as described previously. One unit of β-xylosidase activity was defined as the amount of enzyme required to produce one μmol of *p*-nitrophenol per min at 45°C [15]. Xylanase activity was measured by using the dinitrosalicylic acid reagent for determination of the reducing sugars produced by the enzymatic reaction. One unit of the enzyme was defined as the amount of enzyme required to release 1 μmol of xylose equivalents per min at 45°C [12]. Protein content was determined as described by Lowry *et al.* [11]. Bovine serum albumin was used as a standard protein. Protein concentrations during purification of the enzyme were monitored by measuring the absorbance at 280 nm for estimation of the degree of purification.

Localization of the α-L-Arabinofuranosidase Activity in the *E. coli* Cells Carrying pARK118

The *E. coli* XL1Blue cells carrying pARF118 were grown in LB medium supplemented with 50 μg/ml of ampicillin at 37°C to 1.0 unit at OD_{600} and subsequently followed by an osmotic shock treatment as described by Neu and Heppel [14] with some modifications. A 40 ml of the treated culture was centrifuged at 5,000 ×*g* for 10 min, washed twice in 5 ml of 10 mM Tris buffer (pH 7.0), and suspended in 0.9 ml of 0.58 M sucrose-0.2 mM dithiothreitol-30 mM Tris-HCl buffer (pH 8.0). Conversion to spheroplasts was achieved by addition of 20 μl of lysozyme (2 mg/ml) and 40 μl of 0.1 M EDTA followed by incubation at 23°C for 10 min. The spheroplasts obtained were placed on ice, collected by centrifugation, and the supernatant was retained as the periplasmic fraction. After suspension in 5 ml of 10 mM Tris buffer (pH 7.0), the pellet was sonicated and then centrifuged to separate the soluble (cytoplasmic) fraction from the particulate.

Purification of Arfase from the Recombinant *E. coli* Strain

All purification steps were carried out in 50 mM sodium phosphate buffer (pH 6.5) at 4°C. The *E. coli* cells containing pARK118 were grown in 1-l of LB broth at 37°C for 20 h, harvested by centrifugation (20 min at 20,000 ×*g*), suspended in 500 ml of 50 mM sodium phosphate buffer, and lysed with an ultrasonic homogenizer. After removal of debris by centrifugation (20,000 ×*g*, 20 min), the crude lysate was diluted with 50 ml of the same buffer and fractionated with ammonium sulfate (20-50%). The resulting protein pellet was resuspended in 10 ml of the sodium phosphate buffer. This fraction was desalted by dialysis overnight against 50 mM sodium phosphate buffer (pH 6.5). The dialysate portion (12 ml) was put on a DEAE-Sepharose CL-6B column (2.4×6 cm) equilibrated with 50 mM sodium

phosphate buffer. Proteins were eluted with a linear gradient from 0 to 1 M NaCl in the same buffer at a flow rate of 0.3 ml/min. Active fractions were pooled (5 ml) and ultrafiltered (cutoff 30-kDa) to concentrate the pooled samples to 1 ml. This concentrate was put on a Sephacryl S-300 column (2.2×84 cm) equilibrated with 50 mM sodium phosphate buffer. The elution flow rate of 0.2 ml/min was used, and the eluate was fractionated into 3-ml portions.

Determination of Molecular Mass

For determination of the molecular mass of the purified enzyme, SDS-PAGE was done on a 10% polyacrylamide gel. The native molecular mass of the enzyme was estimated from the results of gel filtration on a Sephacryl S-300 column (Amersham Bioscience). The gel filtration was performed at a flow rate of 0.2 ml/min with 50 mM sodium phosphate buffer (pH 6.5). Molecular mass standards used were phosphorylase B (97,000), bovine serum albumin (68,000), albumin (43,000), and carbonic anhydrase (29,000). The markers for gel filtration were bovine serum albumin (68,000), alcohol dehydrogenase (150,000), and apoferritin (443,000).

Enzymatic Properties

The effects of pH on the enzyme activity were determined at 45°C by using three different buffers: 50 mM sodium acetate (pH 4.5 to 6.5), 50 mM sodium phosphate (pH 6.5 to 7.5), and 50 mM Tris (pH 7.5 to 9.0) buffer. The effects of temperatures on the reaction rate were assessed by performing the standard reaction at various temperatures ranging from 30°C to 60°C. The thermal stability of the enzyme was monitored at 40°C, 50°C, 60°C, and 70°C. After incubation at each temperature for different periods of time, the residual activities were measured under the standard assay conditions.

DNA and Protein Sequencing

DNAs were sequenced with an ABI PRISM Dye Terminator Cycle Sequencing kit using universal or specific primers, and analyzed with an ABI PRISM 310 genetic analyzer (Perkin Elmer, Norwalk, CT, U.S.A.). The N-teminal amino acid sequence of the purified Arfase was determined with a 476A gas-phase protein sequencer (Applied Biosystem, Foster city, CA, U.S.A.).

Total RNA Preparation

E. coli XL1Blue/pARK118 was grown in the basal medium containing 0.5% arabinose to the mid-log phase. The cells were harvested by centrifugation, suspended in 0.1 ml of TE buffer (pH 8.0) containing lysozyme with vigorous voltexing, and then incubated at 37°C for 30 min until the cells were completely lysed. Subsequently, 1 ml of RNA Zol B solution (TX, Friendswood Tel-Test, Inc., U.S.A.) was added, mixed by pippeting, and incubated for 5 min at room temperature. Then, 0.1 ml of chloroform was added, and the reaction mixture was incubated on ice for further 30 min. Following centrifugation at 12,500 rpm for 30 min, the supernatant was transferred to a new 1.5-ml tube containing 0.5 ml of ice-cold isopropanol and incubated at -20°C for 2 h. Total RNA pellet was obtained by centrifugation at 15,000 rpm for 1.5 h, air-dried, suspended in DEPC-treated water, and stored at -70°C for the later use. The concentration of RNA was determined by measuring optical density at 260 nm with a spectrophotometer.

Primer Extension

To prepare the probe, the synthetic primer (5'-CTG CTG TCG GAT GCG AAG GCT CAT AA-3') was labeled with γ-[^{32}P]-ATP (Amersham Bioscience) by using T4 DNA polynucleotide kinase (Takara Shuzo Co., Kyoto, Japen). The purified total RNAs prepared from *E. coli* XL1Blue/pARK118 cells were mixed with 20 µl of the labeled primer and 0.1 volume of 3 M sodium acetate (pH 5.2), and then 2.5 volumes of absolute ethanol was added to the above mixture. After incubation at -20°C for 30 min, the RNAs and the primer were pelleted by centrifugation at 15,000 rpm at 4°C. Dried pellet was suspended in 10 µl of hybridization buffer (50 mM KCl, 25 mM Tris-HCl, pH 8.3). The hybridization mixture was heated at 75°C for 2 min, and then it was slowly cooled down by turning the heating block off. When the temperature dropped to room temperature, the reverse transcripion reaction mixture [1 µl of 0.1 M DTT, 4 µl of 2.5 mM dNTP, 4 µl of 5×M-MLV buffer, and 1 µl of M-MLV (Promega, Madison, WI, U.S.A.) was incubated for 60 min at 42°C. The reaction was terminated by adding 1 µl of 0.5 M EDTA. Residual RNAs were degraded by treatment with 1 µl of pancreatic RNase A. The dried pellet obtained through phenol extraction and subsequent ethanol precipitation was dissolved in loading buffer (80% formamide, 1 mg/ml of bromophenol blue, and 10 mM EDTA , pH 8.0) and stored at -20°C for the later use.

Zymogram

Zymogram was prepared by overlaying the electrophoresed native polyacrylamide gel with a 0.8% agarose gel containing 5 mM 4-methylumbelliferyl-α-L-arabinofuranoside (MUA). Electrophoresis was performed on two 6% nondenaturing polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue R250, and the other gel was washed twice with 50 mM phosphate buffer (pH 6.5). The washed gel was placed on a 0.8% agarose gel containing 5 mM MUA. Following incubation up to 30 min at 45°C, the gel was inspected regularly under UV light to detect the activity bands that are indicative of MUA hydrolysis.

Paper Chromatography

Enzymatic digestion products of arabinoxylan were analyzed by paper chromatography (3 mM Chr, Whatman). The

reaction mixture contained 0.5 ml of 1% (w/v) rye arabinoxylan in 50 mM phosphate buffer (pH 6.5) and 0.5 ml of the enzyme solution (Arfase or xylanase+xylosidase or Arfase+xylanase+xylosidase). After incubation at 45°C for 5 h, the reaction mixture was heated in boiling water for 10 min to stop the reaction. Arabinose and xylose were used as the standards, and the running solvent consisted of *n*-butanol-pyridine-water (6:4:3, v/v). Sugars on the filter were detected by heating at 100°C for a few minutes after spraying with a 1% *p*-anisidine solution [9].

RESULTS AND DISCUSSION

Cloning and Expression of the arfA Gene

Three clones out of 4.2×10^3 white colonies on the selective media showed positive response to pNPAf. Among them, two clones exhibited especially high Arfase activities on pNPAf. Based on the restriction patterns, we concluded that they are the recombinant clones containing the same insert DNA of 5.0 kb. The recombinant plasmid isolated from these clones was named as pARFA. To delimit the

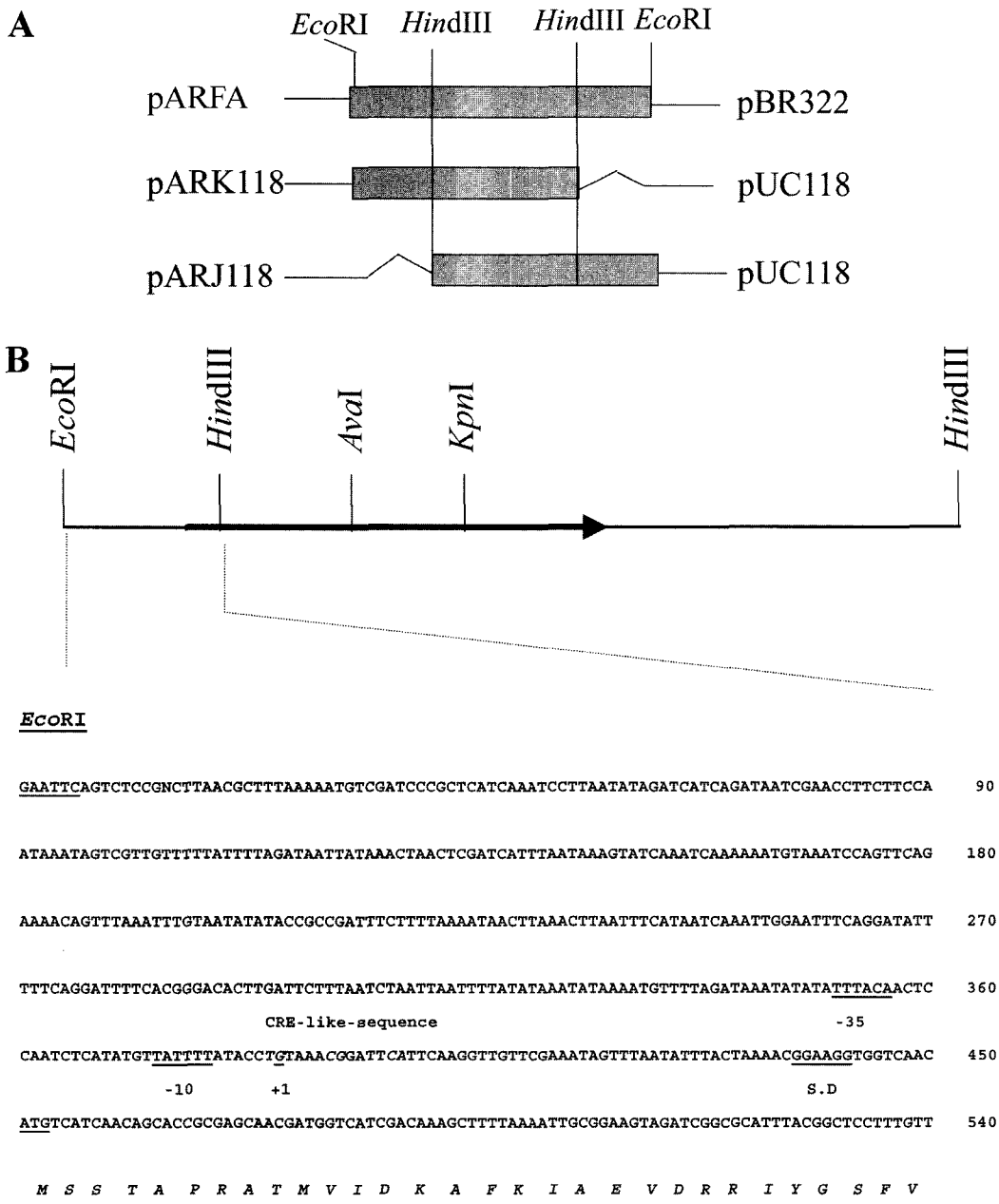

Fig. 1. Description of the pARFA plasmid containing the *arfA* gene and its derivatives, and nucleotide sequence of the *arfA* promoter region.

(A) Location of the *arfA* structural gene on the insert DNA. (B) The putative ribosome binding site (S.D) and the -35 and -10 elements are indicated.

Table 1. Localization of α-arabinofuranosidase activity in the *E. coli* XL1Blue/pARK118 cells.

	Enzyme activity (Unit)	Relative activity (%)
	pARK118	pARK118
Extracellular	55.6	2.15
Periplasmic	10.2	0.39
Cytoplasmic	2527.2	97.46

Localization experiments were performed as described in Materials and Methods.

*arf*A gene in the insert DNA, two deletion constructs, pARK118 and pARJ118, were created from the plasmid pARFA and subjected to assays for Arfase activity. *E. coli* XL1Blue/pARK118 revealed the same level of Arfase activity as *E. coli* XL1Blue/pARFA. But, *E. coli* XL1Blue/pARJ118 showed no detectable Arfase activity (Fig. 1A). These results indicated that the structural gene for Arfase was located within the 3.0 kb *Eco*RI-*Hin*dIII fragment. As expected, the SDS-PAGE analysis identified the presumed ArfA protein of approximately 60 kDa from the cell extracts of *E. coli*/pARFA and *E. coli*/pARK118 (data not shown). In order to determine the subcellular localization of the *arfA* gene product in the *E. coli* XL1Blue cells, the three subcellular fractions prepared from the recombinant *E. coli* cells containing pARK118 were measured for Arfase activity. As shown in Table 1, most of the α-L-arabinofuranosidase activity was detected in the intracellular soluble fraction. This pattern of enzyme distribution was also observed in the original *B. stearothermophilus* No. 236 cells, and could be explained by the amino acid sequence analysis of the Arf A showing no potential signal sequence at the N-terminal region as shown in Fig. 1B. On the other hand, the *E. coli* XL1Blue/pARK118 strain was found to produce a significantly high level of Arfase activity, implying that the cloned *arfA* gene also contained its own strong active promoter in the *E. coli* cells.

Analyses of Nucleotide and Amino Acid Sequences

The nucleotide sequence of the ~3 kb insert DNA in pARK118 was shown to cover the entire Arfase gene and its franking regions. The primary structure analysis of the foreign DNA identified an open reading frame of 1,521 bp that encoded 507 amino acid residues of Arfase. The encoded Arfase has a calculated molecular mass of 57,332 Da and a calculated p*I* of 5.35. At 8 bases upstream of the potential ATG start codon, a putative Shine-Dalgarno (SD) sequence (GGAAGG) could be recognized as shown in Fig. 1B. The putative -35 and -10 regions (TTTACA-17 bp-TATTTT) were also identified in the upstream region of the SD sequence. The translation initiation site described above was confirmed by the N-terminal sequence of the purified enzyme. The N-terminal sequence of Arfase was determined to be X-Ser-Thr-Ala-Pro-Arg-Ala-Thr-Met-Val-Ile-Asp-X-Ala-Phe. This was not a typical signal peptide found in the N-terminal region of the Arfase. To identify the functional promoter among the potential promoters predicted by the computational analysis, the transcription start site was mapped by the primer extension analysis as described in Materials and Methods. A single extension product was detected from the total RNA isolated from the recombinant *E. coli* cells grown in the presence of 0.5% L-arabinose. The size of the extension product indicated that transcription of the *arfA* gene started at the G residue located 65 nt upstream from the Arfase start codon as shown in Fig. 2. As expected, the same transcription start point was identified from the *B. stearothermophilus* No. 236 strain when determined under the same conditions described above (data not shown). These results together suggested that the putative *arfA* promoter is actually active in both *B. stearothermophilus* No. 236 and the *E. coli* recombinant strain. Furthermore, a potential catabolite response element (CRE) was identified in the upstream region of the transcription initiation site, implying that it could act as a cis-element in glucose repression of the *arfA* gene as in the case of the *xynA* gene of *B. stearothermophilus* No. 236 [7]. In fact, the activity of Arfase was observed to be significantly decreased when

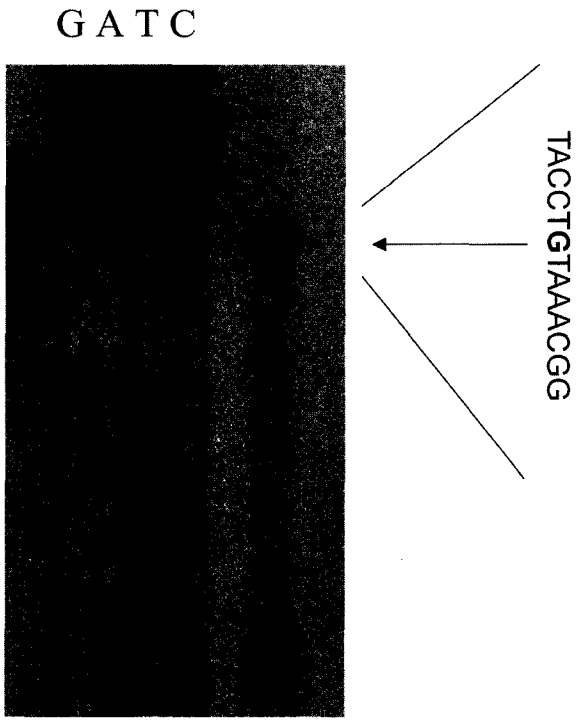

Fig. 2. Primer extension analysis of the *arfA* gene.
Total RNA was prepared from the *E. coli* XL1Blue/pARK118 cells grown in the basal medium containing 0.5% arabinose to mid-log phase. The prepared RNA was hybridized with radiolabeled synthetic primer and extended using M-MLV reverse transcriptase. With the same primer, sequencing was performed according to the Sanger method using Sequenase V.2.0 (USB, U.S.A.).

B. stearothermophilus No. 236 was grown in the basal medium containing 0.5% glucose as a sole carbon source (data not shown). Comparison of the deduced amino acid sequences showed that the Arfase of *B. stearothermophilus* No. 236 had 71.34%, 70.16%, and 68.58% identities with those of *B. stearothermophilus* T-6, *B. halodurans*, and *B. subtilis*, respectively. In addition, previous studies have proposed that the glycoside hydrolase family 51(GH51) α-L-arabinofuranosidases have highly conserved two glutamic acid residues, comprising the key catalytic acid/base and nucleophile residues [24]. For example, in *Geobacillus stearothermophilus* T-6, Glu-175 and Glu-294 were found to have the roles of the acid-base catalytic residue and the key catalytic nucleophile residue, respectively [5]. Therefore, we speculate that the two conserved Glu residues, Glu-196 and Glu-323, in the Arfase of *B. stearothermophilus* No. 236 as indicated in Fig. 3 may also play the same roles in the catalytic reaction.

Purification of the Recombinant α-L-Arabinofuranosidase

Arfase was purified from *E. coli* XL1Blue/ pARK118 as described in Materials and Methods. The purification procedure of Arfase from the recombinant *E. coli* cells is summarized in Table 2, and the SDS-PAGE results obtained during the purification steps are shown in Fig. 4A. The final-step fraction gave a single band by SDS-PAGE when stained with Coomassie Brilliant blue. The purified Arfase exhibited the specific activity of 26.11 U/mg at 45°C with the reduced pNPAf as the substrate. Analyses of the purified enzyme by SDS-PAGE (Fig. 4B) and gel filtration on Sephacryl 300 column (data not shown) revealed that molecular masses of the Arfase were 60 and 190 kDa, respectively, indicating that the functional Arfase had a trimeric structure. Interestingly, this trimeric subunit structure is not common among bacterial Arfases [18].

Next, to confirm the activity of the purified recombinant Arfase, a zymogram was obtained as described in Materials

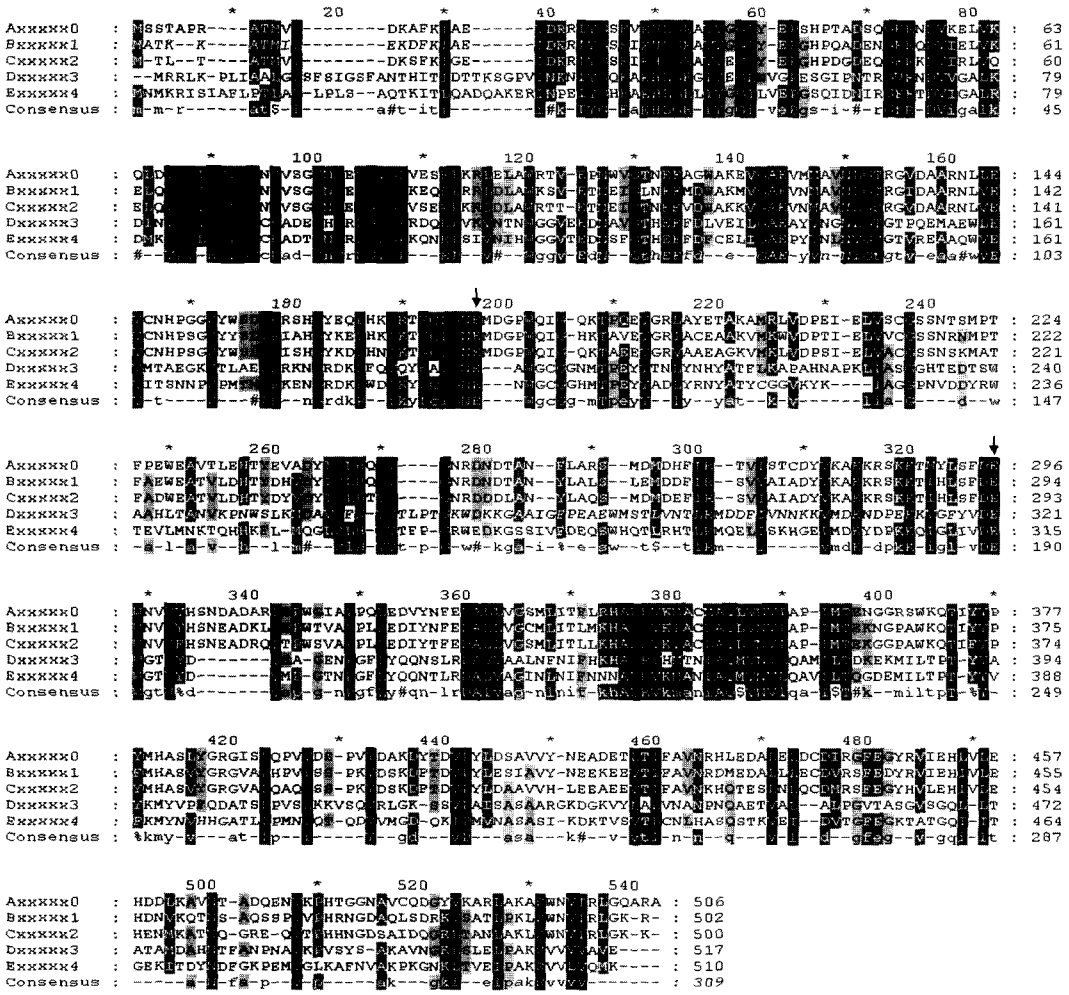

Fig. 3. Multiple sequence alignment of the α-L-arabinofuranosidases from various origins.
A: *B. stearothermophilus* No. 236; B: *B. stearothermophilus* T-6; C: *B. halodurans*; D: *Pseudomonas cellulosa*; E: *Cytophago xylanolytica* arabinofuranosidase II.

Table 2. Summary of the purification results of α-L-arabinofuranosidase from *E. coli* XL1Blue/pARK118.

Purification steps	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery (%)	Purification
Cytoplasmic extract	446.5	3298.0	7.39	100.00	1.00
Ammonium sulfate fractionation	198.9	2051.4	10.31	62.2	1.40
DEAE- Sepharose CL-6B	3.56	77.98	21.90	2.36	2.96
Sephacryl S-300	1.23	32.12	26.11	0.97	3.53

and Methods. As shown in Fig. 5, a single activity band migrating to a position that was identical to the position of the protein band with molecular mass of about 190 kDa was detected from both the crude extract of the *E. coli* XL1Blue/pARK118 cells and the purified Arfase fraction. This result provides clear evidence that the cloned DNA in pARK118 contains the gene encoding the Arfase of *B. stearothermophilus* No. 236.

Enzymatic Properties

Enzymatic properties were investigated with the purified enzyme prepared as described above. The experimental measurements of the V_{max} and K_m values were determined by Lineweaver-Burk plot of the enzyme kinetics. With pNPAf as the substrate, the enzyme exhibited a K_m value of 1.19 mM and a V_{max} of 26.1 U/mg. The optimal temperature and pH were determined to be 55°C and 6.5, respectively. This optimum pH is typical for Arfases from bacteria, whereas fungi produce the enzymes with a more acidic pH optimum [8]. The Arfase was relatively stable up to 60°C; about 55% of its initial full activity was retained after incubating at 60°C for 6 h but the activity was rapidly decreased at 70°C, exhibiting higher thermal stability than those from other organisms (Fig. 6) [8]. This high thermostability of the Arfase of *B. stearothermophilus* No. 236 can be a great advantage of the enzyme for its applifications in xylan hydrolysis.

α-L-Arabinofuranosidase in Xylan Degradation

To understand the actual contribution of Arfase to the hydrolysis of xylan, we mixed the enzyme with endoxylanase and/or β-xylosidase, and determined any changes in the hydrolysis rates caused by the Arfase addition by measuring the release of xylose equivalents from the xylan substrates (Table 3). Endoxylanase and β-xylosidase showed a cooperative action on all the substrates tested in this experiment, illustrating that β-xylosidase increased reducing sugar production by hydrolyzing xylooligomers, the products of endoxylanase action, and possibly this hydrolysis, in turn, relieved the end-product inhibition imposed upon endoxylanase. As shown in Table 3, β-xylosidase supported a higher level of hydrolysis of birchwood xylan than xylanase did. This unsuspected result may be, at least in part, due to the unusual nature of the *B. stearothermophilus* No. 236 β-xylosidase; the enzyme has been demonstrated to function extraordinarily as an exoxylanase which has been identified only in fungi [13].

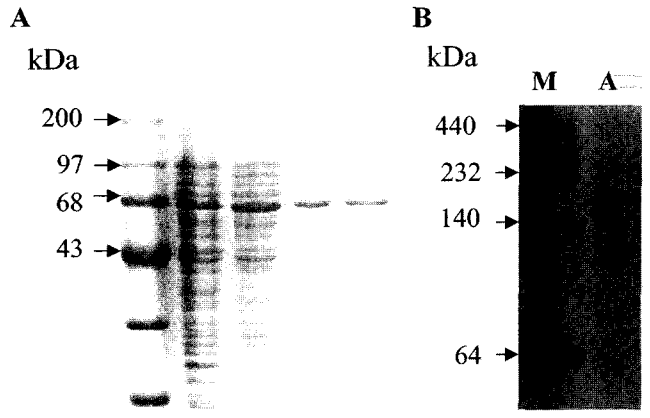

Fig. 4. SDS-PAGE and native PAGE of the α-L-arabinofuranosidase from *E. coli* XL1Blue/pARK118.
(A) 10% SDS-PAGE of the α-L-arabinofuranosidase at purification steps. Lane 1, Size markers; lane 2, Crude extract of *E. coli* XL1blue/pARK118; lane 3, 20- 50% ammonium sulfate precipitate; lane 4, DEAE sepharose CL-6B pool; lane 5, Sephacryl S-300 pool. (B) 6% Native PAGE of the α-L-arabinofuranosidase. Lane M, MW standards; lane A, the purified α-L-arabinofuranosidase. The standards were ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa).

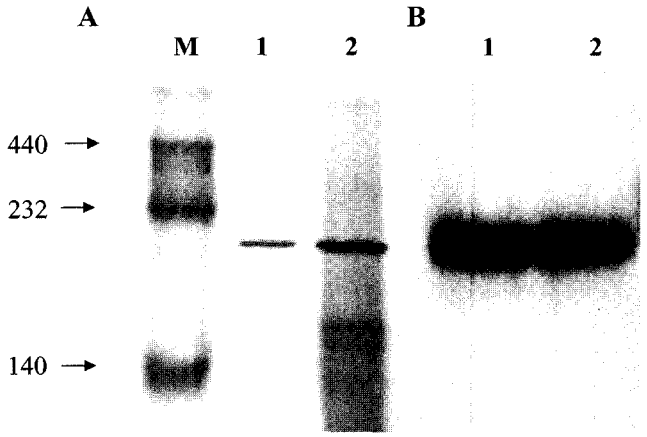

Fig. 5. Zymogram and protein staining of a native PAGE gel for the α-L-arabinofuranosidase from *E. coli* XL1Blue/pARK118.
(A) 6% native PAGE gel. Lane 1, Sephacryl 300 pool; lane 2, crude extract of the *E. coli* XL1Blue/pARK118 strain; lane M, size marker. (B) MUA zymogram showing the Arfase activity as UV-fluorescent bands. Lane 1, Sephacryl 300 pool; lane 2, crude extract of the *E. coli* XL1Blue/pARK118 strain.

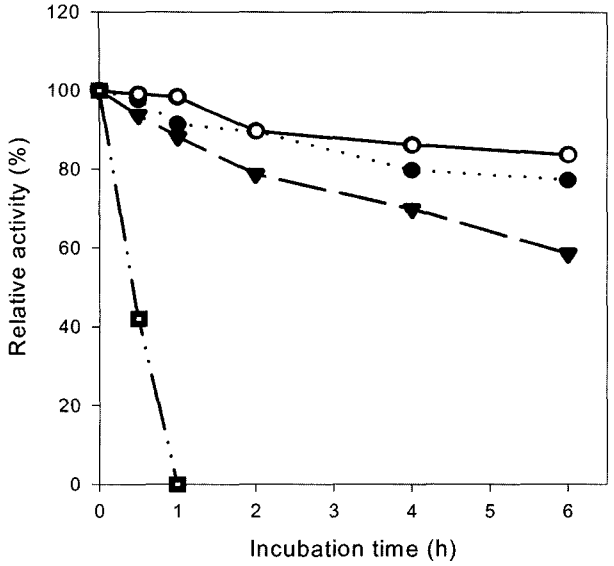

Fig. 6. Thermostability of the α-L-arabinofuranosidase.
Enzyme solutions were incubated at the various temperatures (□, 70°C; ▼, 60°C; ●, 50°C; ○, 40°C) up to 6 h. Residual enzyme activities were measured with 1 h intervals at 45°C for 20 min, and represented by the percentage of the untreated control.

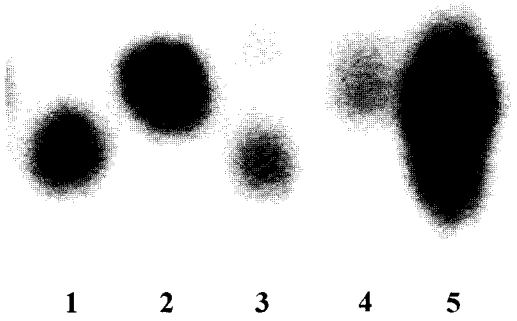

Fig. 7. Paper chromatogram of hydrolysis products of arabinoxylan.
The paper was developed twice by the ascending method with the solvent system; *n*-butanol-pyridine-water (6:4:3). The reaction mixture contained 0.5 ml of 1% arabinoxylan in 50 mM phosphate buffer (pH 6.5) and the same volume of enzyme solution. 1, Authentic arabinose; 2, authentic xylose; 3, the reaction mixture with α-L-arabinofuranosidase; 4, the reaction mixture with endoxylanase and β-xylosidase; 5, the reaction mixture with endoxylanase, β-xylosidase, and α-L-arabinofuranosidase. The amounts of enzymes used in the reactions are as follows; endoxylanase (14.4 U), α-L-arabinofuranosidase (43.2 U), and β-xylosidase (28.8 U). Numbers indicate sugar concentrations (mg/ml) measured by a densitometer.

As expected, the α-L-arabinofuranosidase also exhibited remarkable synergistic effects with endoxylanase and β-xylosidase on rye arabinoxylan. No obvious synergism was detected on oat spelt xylan and especially on birchwood xylan, which is known to be composed of only trace amounts of the arabinose side chain [6]. This is probably due to removal of the side chains by Arfase, which results in relieving the steric hindrance and exposing additional sites at which endoxylanase and β-xylosidase can act. Taken together, these results reinforced that the cooperative action of the three xylanolytic enzymes, endoxylanase, β-xylosidase, and α-L-arabinofuranosidase, is essential for complete hydrolysis of the xylan substrates, especially containing large amounts of arabinose side chains. Figure 7 shows the paper chromatogram, thus confirming the results described in Table 3. The Arfase or the endoxylanase and β-xylosidase mixture alone released

Table 3. Cooperative action of α-L-arabinofuranosidase, β-xylosidase, and endoxylanase on birchwood, oat spelt, and arabinoxylan.

	Reducing sugar yields (μg of xylose equivalents)		
	Birchwood[a]	Oat spelts[b]	Arabinoxylan[c]
Xylanase	50.6±1.5	40.2±1.8	43.3±1.7
Xylosidase	60.2±1.6	26.1±2.0	22.2±2.1
Arabinofuranosidase	ND*	ND	30.1±1.8
Arabinofuranosidase & xylanase	55.0±4.8	40.8±1.7	80.2±1.0
Arabinofuranosidase & xylosidase	59.7±3.2	28.2±2.0	60.7±1.39
Xylosidase & xylanase	143.6±15.8	106.9±13.4	58.9±1.42
Arabinofuranosidase, xylosidase, & xylanase	152.77±14.9	135.95±14.7	139.5±16.7

All reactions were performed at 45°C for 20 min. Reducing sugar released was quantitated by using the DNS method. Reaction mixtures contained 0.5 ml of xylan substrate and the same volume of endoxylanase, β-xylosidase, and/or α-L-arabinofuranosidase, alone or in combinations.
[a]The enzymes used in the reactions were endoxylanase (0.03 U), α-L-arabinofuranosidase (11.9 U), and/or β-xylosidase (1.7 U), and birchwood xylan was used as the substrate.
[b]The enzymes used were endoxylanase (0.03 U), α-L-arabinofuranosidase (11.9 U), and/or β-xylosidase (1.7 U), and 0.5% oat spelt xylan was used as the substrate.
[c]The enzymes used were endoxylanase (0.03 U), α-L-arabinofuranosidase (17.3 U), and/or β-xylosidase (3.4 U), and 1% arabinoxylan was used as the substrate.
*ND: Not Detected.

small amounts of arabinose and xylose from arabinoxylan. In contrast, the mixture of the three enzymes produced significantly more arabinose and xylose.

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