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Characterization of a GH36 β-L-Arabinopyranosidase in *Bifidobacterium adolescentis*

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Abstract: β-L-Arabinopyranosidases are classified into the glycoside hydrolase family 27 (GH27) and GH97, but not into GH36. In this study, we first characterized the GH36 β-L-arabinopyranosidase BAD_1528 from *Bifidobacterium adolescentis* JCM1275. The recombinant BAD_1528 expressed in *Escherichia coli* had a hydrolytic activity toward *p*-nitrophenyl (*p*NP)-β-L-arabinopyranoside (Ara*p*) and a weak activity toward *p*NP-α-D-galactopyranoside (Gal). The enzyme liberated L-arabinose efficiently not from any oligosaccharides or polysaccharides containing Ara*p*-β1,3-linkages, but from the disaccharide Ara*p*-β1,3-L-arabinose. However, we were unable to confirm the *in vitro* fermentability of Ara*p*-β1,3-Ara in *B. adolescentis* strains. The enzyme also had a transglycosylation activity toward 1-alkanols and saccharides as acceptors.

Key words: β-L-arabinopyranosidase, glycoside hydrolase family 36, arabinogalactan protein, *Bifidobacterium adolescentis*, transglycosylation

INTRODUCTION

L-Arabinose is a widely occurring sugar in plant cell wall polysaccharides and proteoglycans such as arabinan, arabinoxylan, pectic arabinogalactan, arabinogalactan protein (AGP), and extensin, and the L-arabinose content is in the range of 5–10 % of the cell wall saccharides in *Arabidopsis thaliana* and *Oryza sativa*.[1)] Although the pyranose ring form is thermodynamically more stable than the furanose form, α-L-arabinofuranose (α-L-Ara*f*) is the primary form in these polysaccharides. The presence of β-L-arabinopyranose (β-L-Ara*p*) has been reported in AGPs from wheat flour,[2)] larch,[3)] and gum arabic,[4)] which is substituted with α-L-Ara*f* residues attached to the β-1,6-linked galactan side chain as the structural motif of β-Ara*p*-1,3-α-Ara*f*-1,3-. The terminal β-Ara*p* content is in the range of 26–27 % of the total L-arabinose in larch arabinogalactans (AGs).[3)5)] In the case of wheat flour AGP, β-L-Ara*p* is directly attached to the β-1,6-linked galactan side chain.[2)]

β-L-Arabinopyranosidases (EC 3.2.1.88) catalyze the removal of a terminal β-L-Ara*p* residue from the nonreducing end of its substrate and are included in glycoside hydrolase family 27 (GH27) and GH97 in the CAZy database. In the case of GH27, β-L-arabinopyranosidases are obtained from *Streptomyces avermitilis*,[6)] *Fusarium oxysporum,*[7)] *Chitinophaga pinensis*,[8)] *Geobacillus stearothermophilus*,[9)] and *A. thaliana*.[10)] We also cloned and characterized GH27 β-L-arabinopyranosidase (BLLJ_1823) from *Bifidobacterium longum* subsp. *longum*.[11)] GH97 β-L-arabinopyranosidase has recently been cloned from *Bacteroides thetaiotaomicron*.[12)] Because of the structure similarity between β-L-Ara*p* and α-D-galactopyranose (α-Gal), α-D-galactopyranosidase (EC 3.2.1.22) can potentially possess a β-L-arabinopyranosidase activity. In fact, almost all β-L-arabinopyranosidases belonging to GH27 and GH97 have α-D-galactopyranosidase activity.

On the other hand, α-D-galactopyranosidases are included in GH4, GH27, GH36, GH57, GH97, and GH110. In particular, GH36 α-D-galactopyranosidases have been characterized in several bacteria, fungi, and plants. However, the weak β-L-arabinopyranosidase activity (the K_m for *p*NP-β-Ara*p* is 500-fold higher than that for *p*NP-α-Gal) was reported only in the GH36 from the archaeon *Sulfolobus solfataricus*.[13)] GH36 α-D-galactopyranosidases have been cloned and characterized in several bifidobacterial species such as *B. longum*,[14)] *B. adolescentis*,[15)] *B. bifidum*,[16)] and *B. breve*.[17)] These enzymes also have transglycosylation activities for the synthesis of neo-glycoconjugates. *B. adolescentis* JCM1275 encodes BAD_1528, which is another member of GH36 with 24 % identity of a characterized GH36 α-D-galactopyranosidase BAD_1576. In this study, we characterized BAD_1528 from *B. adolescentis* JCM1275 as a novel GH36 β-L-arabinopyranosidase.

MATERIALS AND METHODS

Materials. Gum arabic and *p*-nitrophenyl (*p*NP) substrates were both obtained from Sigma-Aldrich (St. Louis, MO,

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Abbreviations: AGP, arabinogalactan protein; AG, arabinogalactan; Ara*p*, L-arabinopyranose; Ara*f*, L-arabinofuranose; Ara*p*-β1,3-Ara, β-Ara*p*-1,3-L-arabinose; Gal-α1,3-Ara, α-Gal-1,3-L-arabinose; Ara*p*-β1,3-Ara*f*-Gal_3, β-Ara*p*-1,3-α-Ara*f*-1,3-β-Gal-1,6-β-Gal-1,6-Gal; Ara*p*-β1,3-ART, β-Ara*p*-1,3-L-arabinitol; *p*NP, *p*-nitrophenyl; GH, glycoside hydrolase family; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PYF, peptone-yeast extract-Fildes.

USA). Larch AG was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Wheat flour arabinoxylan and sugar-beet arabinan were obtained from Megazyme (Wicklow, Ireland). These polysaccharides were used after purification by ethanol precipitation. β-Ara*p*-1,3-α-Ara*f*-1,3-β-Gal-1,6-β-Gal-1,6-Gal (Ara*p*-β1,3-Ara*f*-Gal_3) was prepared from larch AG as previously described.[18] β-Ara*p*-1,3-L-arabinose (Ara*p*-β1,3-Ara) and α-Gal-1,3-L-arabinose (Gal-α1,3-Ara) were prepared by the partial acid hydrolysis of larch AG and gum arabic, respectively.[5][19] All other chemicals were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ara*p*-β1,3-L-arabinitol (Ara*p*-β1,3-ART) was prepared from Ara*p*-β1,3-Ara by reduction with sodium borohydride.

Expression and purification of recombinant BAD_1528. The genomic DNA of *B. adolescentis* JCM1275 was used for the PCR amplification of the BAD_1528 gene. The forward (5′- AGGAGATATACCATGATCAGGCGGTATCT-3′) and reverse (5′-GTGGTGGTGCTCGAGGCGGATGGCGAAGATGC-3′) primers were designed from nucleotides 1–17 and 2387–2403, respectively. The underlined letters represent the nucleotides complementary to the template. The amplicon was subsequently cloned into the pET-23d vector (Novagen Inc., Madison, WI, USA) using an In-Fusion HD cloning kit (Clontech Laboratories Inc., Palo Alto, CA, USA). This nucleotide sequence reported in this paper has been deposited in the DDBJ/GenBank/EMBL Data Bank with accession number LC374389. *E. coli* SoluBL21 cells (Genlantis, Inc., San Diego, CA, USA) were transformed with the plasmid and subsequently grown at 37 °C using the Overnight Express Autoinduction System (Novagen). The cell culture was subsequently centrifuged, and the resultant pellet was then resuspended in the BugBuster protein extraction reagent (Novagen). The His-tagged BAD_1528 protein was purified with a column containing the TALON metal affinity resin (Clontech). The 10 mM imidazole fraction containing the eluted protein was desalted and concentrated using an ultrafiltration membrane with a 10 kDa cutoff (Millipore Co., Billerica, MA, USA).

Enzyme assays. The hydrolytic activity of the BAD_1528 enzyme was assayed using *p*NP-β-Ara*p* as the standard substrate. The 200 μL reaction mixture containing BAD_1528 and 1 mM substrate in 50 mM sodium acetate buffer (pH 5.5) was incubated at 40 °C for 20 min. The enzymatic reaction was terminated by adding 300 μL of 1 M sodium carbonate. The absorbance of the released *p*NP was measured at 400 nm. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of *p*NP per minute.

The substrate specificity toward each *p*NP substrate was analyzed as follows: Ten mM substrate was incubated at 40 °C for 16 h with 100 mU/mL of BAD_1528 in 40 μL of 50 mM sodium acetate buffer (pH 5.0). The reaction products were spotted on a silica gel 60 aluminum plate (Merck KGaA, Darmstadt, Germany) using a 7:1:2 (v/v/v) *n*-propanol/ethanol/water solvent mixture. The sugars were visualized by spraying orcinol-sulfate reagent on the plate.[20]

The specific activities toward the *p*NP substrates, disaccharides, and oligosaccharides as listed in Table 2 were analyzed as follows: Each substrate was incubated with 300 mU/mL BAD_1528 in 40 μL of 50 mM sodium acetate buffer (pH 5.0). After incubating the reaction mixture at 40 °C for suitable time, the reaction was stopped by boiling this mixture for 3 min. The reaction mixtures were assessed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using galactose and L-arabinose as the standards. The saccharides were analyzed using a CarboPac PA-1 column (4 mm internal diameter × 250 mm; Dionex Corp., Sunnyvale, CA, USA). The column was eluted at a flow rate of 1.0 mL/min using the following gradient: 0–5 min, 100 % eluent A (0.1 M NaOH); 5–30 min, 0–100 % eluent B (0.5 M sodium acetate and 0.1 M NaOH); and 30–35 min, 100 % eluent B.

The pH dependence of enzyme activity was determined using *p*NP-β-Ara*p* as the substrate between pH 3.5 and 7.0 using the following buffers: 50 mM sodium acetate (pH 3.5–6.0) and 50 mM MES (pH 5.5–7.0). The effect of temperature on enzyme activity was determined using 50 mM sodium acetate buffer (pH 5.0) at 25–50 °C.

Kinetic analysis. The kinetic parameters for BAD_1528 were determined using 0.5–8.0 mM *p*NP-β-Ara*p*, *p*NP-α-Gal, and Ara*p*-β1,3-Ara. The reactions with each *p*NP substrate were measured as *p*NP concentrations by measuring the absorbance of the reaction mixture at 400 nm, and that with Ara*p*-β1,3-Ara was measured as L-arabinose concentration by HPAEC-PAD as described above.

Transglycosylation activities. The transglycosylation reactions were performed using *p*NP-β-Ara*p* as the donor and 1-alkanols, monosaccharides, disaccharide, and *p*NP-β-Ara*p* as acceptors. For the transglycosylation reactions in the presence of 1-alkanols, 2.5 mM *p*NP-β-Ara*p* was incubated at 40 °C for 2 h with 100 mU/mL of BAD_1528 in 40 μL of 50 mM sodium acetate buffer (pH 5.0) with 10 % methanol, ethanol, or 1-propanol as the acceptor. Subsequently, the reaction products were analyzed by TLC. The transglycosylation product in the presence of methanol was purified with activated carbon (Autoprep fiberAC, Showa Denko K.K., Tokyo, Japan) and analyzed by HPAEC-PAD. For the transglycosylation reactions in the presence of monosaccharides and disaccharide, 2.5 mM *p*NP-β-Ara*p* was incubated at 40 °C for 16 h with 40 mU/mL of BAD_1528 in 1,000 μL of 50 mM sodium acetate buffer (pH 5.0) with 300 mM glucose, galactose, L-arabinose, or sucrose as the acceptor. The reaction products were purified with activated carbon and analyzed by HPAEC-PAD. For the transglycosylation reactions in the presence of *p*NP-β-Ara*p*, 55 mM *p*NP-β-Ara*p* was incubated at 40 °C for 5 h with 100 mU/mL of BAD_1528 in 800 μL of 50 mM sodium acetate buffer (pH 5.0). The reaction product was purified by HPLC with a Cosmosil PBr column (10 mm internal diameter × 250 mm; Nacalai Tesque Inc., Kyoto, Japan) at 30 °C. The column was eluted at a flow rate of 4.7 mL/min using the following gradient: 0–2 min, 100 % eluent A (water); 2–30 min, 0–100 % eluent B (90 % acetonitrile in water). The elution was monitored using a UV detector (UV-2070; JASCO Corporation, Tokyo, Japan) at 254 nm. Subsequently, the fractions containing the transglycosyla-

tion product were collected and analyzed by TLC, HPAEC-PAD, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics GmbH, Leipzig, Germany).

Assays of bacterial enzyme activities and in vitro fermentability of Arap-β1,3-Ara. *B. adolescentis* JCM1275 and JCM15918 were cultured at 37 °C under anaerobic condition using the AnaeroPack system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) in a modified MRS broth containing 2 % glucose or galactose as the carbohydrate source and 0.05 % L-cysteine hydrochloride. The cell cultures were centrifuged at 10,000 rpm for 10 min, and the resultant pellets were washed with 50 mM sodium acetate buffer (pH 5.5). Afterwards, the pellets were sonicated with a Branson Sonifier 250 (Branson Ultrasonics Corporation, Danbury, CT, USA). The cell lysates were centrifuged at 15,000 rpm for 10 min, and the supernatants were incubated with *p*NP-β-Ara*p* or Ara*p*-β1,3-Ara at 37 °C for 16 h and then analyzed by TLC and HPAEC-PAD as described above.

The *in vitro* fermentabilities of Ara*p*-β1,3-Ara were examined on peptone-yeast extract-Fildes (PYF) medium containing 0.2 % Ara*p*-β1,3-Ara. The growth profiles of *B. adolescentis* JCM1275 and JCM15918 were monitored by measuring the optimal density at 600 nm and also the pH profile.

RESULTS

Sequence analysis and phylogenetic relationship of BAD_1528.

BAD_1528 lacks the putative signal peptide and terminal transmembrane domain, according to the signalP 4.1 and InterPro servers, suggesting that the enzyme localizes to the intracellular compartment. The genes homologous to BAD_1528 were conserved in several bifidobacterial species such as *B. animalis* subsp. *lactis* (59 %), *B. catenulatum* (81 %), *B. pseudocatenulatum* (81 %), *B. dentium* (75 %), *B. kashiwanohense* (81 %), and *B. pseudolongum* (58 %). The catalytic region of BAD_1528 were phylogenetically independent of other characterized GH36 α-D-galactopyranosidases (21–27 % identities) and was located far (<20 % identities) from the GH27 enzymes having β-L-arabinopyranosidase/α-D-galactopyranosidase activities (Fig. 1A). BAD_1528 formed a gene cluster with several L-arabinosidase candidates belonging to GH27, GH43, GH51, and GH127 (Fig. 1B). *B. adolescentis* JCM15918, BBMN23, and 22L conserved these L-arabinosidase candidates except GH27 β-L-arabinopyranosidase. On the other hand, *B. longum* did not conserved BAD_1528 homologous gene, and other bifidobacterial species conserved BAD_1528 homologous gene and part of L-arabinosidase candidates.

Preparation and characterization of recombinant BAD_1528.

The recombinant BAD_1528 protein was expressed at 37 °C as a soluble protein. SDS-PAGE showed that the purified recombinant BAD_1528 protein migrated as a single band with an apparent molecular mass of 89 kDa (Fig. 2), which was in agreement with its calculated molecular mass of 88,803 Da.

The substrate specificities of BAD_1528 were identified using synthetic *p*NP substrates. BAD_1528 hydrolyzed *p*NP-β-Ara*p* and weakly hydrolyzed *p*NP-α-Gal, but not other *p*NP substrates (Fig. 3). The optimal temperature and pH for *p*NP-β-Ara*p* were 40 °C and 5.0, respectively (Fig. 4). Several disaccharides, oligosaccharides, and polysaccharides were used to identify the specificities of BAD_1528. Ara*p*-β1,3-Ara was best substrate for the enzyme, except for *p*NP substrates (Fig. 5A and Table 1). BAD_1528 had weak activities for Ara*p*-β1,3-ART and Gal-α1,3-Ara (Figs. 5B and C, respectively). These specific activities were 0.0820 and 0.177 % of those for Ara*p*-β1,3-Ara, respectively (Table 2). On the other hand, the hydrolysis of Ara*p*-β1,3-Ara*f*-Gal_3 was below the detection limit after 16 h (Fig. 5D). The enzyme also had no activities for all polysaccharides including gum arabic, larch AG, arabinoxylan, and arabinan (data not shown). The K_m (1.48 mM) for *p*NP-β-Ara*p* was 5.9-fold and 4.0-fold lower than those for Ara*p*-β1,3-Ara and *p*NP-α-Gal, respectively, and the k_{cat} (81.5 s^{-1}) for *p*NP-β-Ara*p* was 11-fold and 34-fold higher than those for Ara*p*-β1,3-Ara and *p*NP-α-Gal, respectively (Table 1). Consequently, the k_{cat}/K_m values for Ara*p*-β1,3-Ara and *p*NP-α-Gal were 62-fold and 135-fold lower than those for *p*NP-β-Ara*p*, respectively.

Transglycosylation activity of recombinant BAD_1528.

When 10 % methanol and ethanol were used as the acceptors, the transglycosylation products were detected by TLC (Fig. 6A). On the other hand, the transglycosylation product was not confirmed with 10 % 1-propanol as an acceptor. The purified transglycosylation product to methanol was partially hydrolyzed to L-arabinose by BAD_1528 treatment (Fig. 6B), which indicates that the transglycosylation product is methyl-β-Ara*p* with the retention of the anomeric configuration.

Next, glucose, galactose, L-arabinose, sucrose, and *p*NP-β-Ara*p* were used as the acceptors for the transglycosylation reactions. The arabinopyranosyl moiety was slightly transferred to all the acceptors (data not shown), and the partial purified products were hydrolyzed by BAD_1528 treatment (Fig. 7), which indicate that the arabinopyranosyl moieties were linked by the β-anomeric form. These data suggest that BAD_1528 has broad acceptor specificities, and that the arabinopyranosyl unit is not exclusively attached by β1,3-linkage. Especially, purified Ara*p*-β-Ara*p*-β-*p*NP was detected at 13.3 min on HPAEC-PAD (Fig. 7E), which is different from the peak corresponding to *p*NP-β-Ara*p* (retention time: 15.0 min). MALDI-TOF MS revealed a molecular ion peak at *m/z* 405.05 for Ara*p*-β-Ara*p*-β-*p*NP (calc. *m*/*z* 403.11). Ara*p*-β-Ara*p*-β-*p*NP had a high specificity toward BAD_1528, which was 56.6 % of that for Ara*p*-β1,3-Ara (Table 2). Almost all GH36 α-D-galactopyranosidases have transglycosylation activities for the synthesis of α-galactosyl neo-glycoconjugates.[14)15)17)] Therefore, BAD_1528 has a potential for the production of β-L-arabinopyranosyl neo-glycoconjugates.

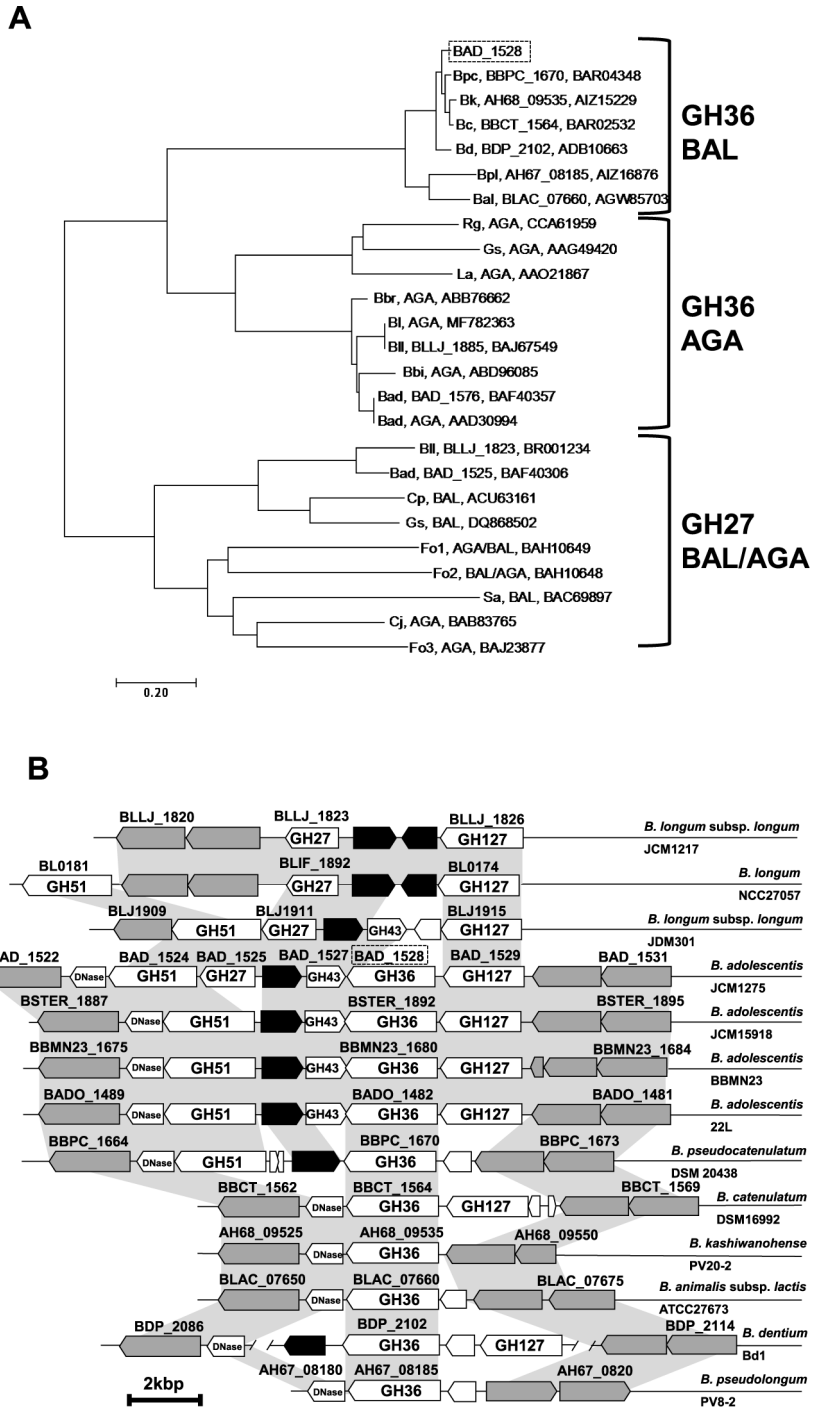

Fig. 1. Phylogenetic relationships and gene clusters of BAD_1528.

A, The phylogenetic tree of BAD_1528 with homologous proteins was constructed by the neighbor-joining method using the aligned sequences with the MUSCLE program implemented in MEGA7 software. The locus tag or the characterized enzymatic activities are shown with the abbreviations of the organisms and the GenBank accession numbers. The protein characterized in this study is enclosed in the dash box. The abbreviations of the enzymatic activities and the organisms are as follows: AGA, α-D-galactopyranosidase; BAL, β-L-arabinopyranosidase; Bad, *B. adolescentis*; Bal, *B. animalis* subsp. *lactis*; Bbi, *B. bifidum*; Bbr, *B. breve*; Bc, *B. catenulatum*; Bd, *B. dentium*; Bk, *B. kashiwanohense*; Bl, *B. longum*; Bll, *B. longum* subsp. *longum*; Bpc, *B. pseudocatenulatum*; Bpl, *B. pseudolongum*; Cj, *Clostridium josui*; Cp, *Chitinophaga pinensis*; Fo, *Fusarium oxysporum*; Gs, *Geobacillus stearothermophilus*; La, *Lactobacillus acidophilus*; Rg, *Ruminococcus gnavus*; Sa, *Streptomyces avermitilis*. B, The gene clusters encoding L-arabinosidases in *Bifidobacterium* strains. Genes encoding sugar transporters and transcriptional regulators are depicted in gray and black arrows, respectively. Light gray bars indicate orthologous regions. The white arrows indicate glycoside hydrolase candidates or hypothetical proteins. The numbers of predicted glycoside hydrolases or the predicted enzymatic activities are shown in the white arrows with locus tags. DNase, TatD DNase family protein.

Bacterial enzyme activities and in vitro fermentability of Arap-β1,3-Ara.

When cell lysates of *B. adolescentis* JCM1275 and JCM15918 grown in the medium containing glucose and galactose were used as the enzyme source, β-L-arabinopyranosidase activities were observed using *p*NP-β-Ara*p* (Fig. 8). Moreover, weak activities for Ara*p*-β1,3-Ara were detected by TLC (Fig. 8), and the released L-arabinose was

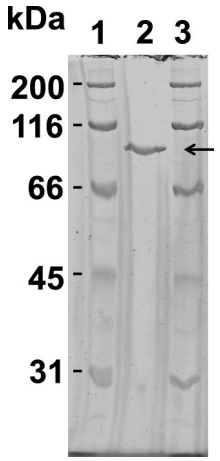

Fig. 2. SDS-PAGE analysis of recombinant BAD_1528.
Purified BAD_1528 was electrophoresed on a 10 % polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lanes 1 and 3, molecular size markers; lane 2, purified enzyme. The arrow indicates the band that corresponds to BAD_1528.

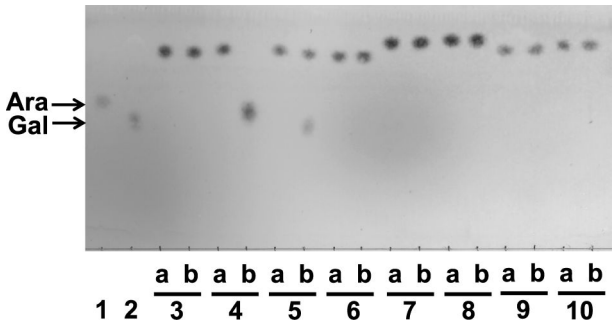

Fig. 3. TLC analysis of BAD_1528 reactions with the *p*NP-substrates.
The *p*NP-substrates were incubated in the absence (lane a) or presence (lane b) of the recombinant enzyme at 40 °C for 16 h. *p*NP-α-Ara*p* (lane 3), *p*NP-β-Ara*p* (lane 4), *p*NP-α-Gal (lane 5), *p*NP-β-Gal (lane 6), *p*NP-α-Xyl (lane 7), *p*NP-β-Xyl (lane 8), *p*NP-α-Glc (lane 9), and *p*NP-β-Glc (lane 10) were used as substrates. Lane 1, L-arabinose standard; lane 2, galactose standard.

detected by HPAEC-PAD (data not shown). Although we predicted that *B. adolescentis* JCM1275 and JCM15918 possess Ara*p*-β1,3-Ara assimilation ability, we observed no distinct differences in growth between culture mediums without any carbohydrate source and with Ara*p*-β1,3-Ara (data not shown). Moreover, no decrease in the amount of Ara*p*-β1,3-Ara in the culture medium was detected by HPAEC-PAD (data not shown). These data suggested that Ara*p*-β1,3-Ara is not degraded or metabolized in *B. adolescentis* strains used in this study.

DISCUSSION

In the present study, we clarified BAD_1528 to be the first characterized GH36 β-L-arabinopyranosidase that exhibits a hydrolytic activity toward Ara*p*-β1,3-Ara derived from a natural substrate. The enzyme showed little or no releasing activities toward oligosaccharides or polysaccharides containing Ara*p*-β1,3-Ara*f*-structures and had a weak activity for Ara*p*-β1,3-ART. Because the pyranose ring form is thermodynamically more stable than the furanose

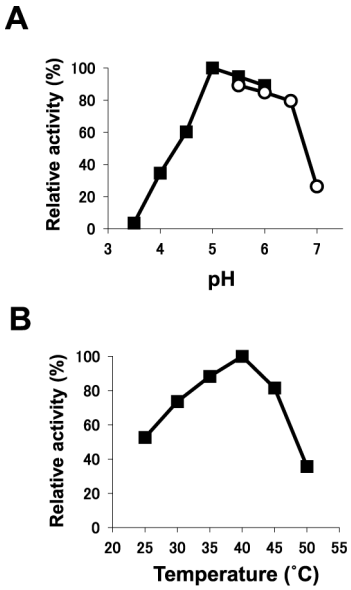

Fig. 4. Effects of pH and temperature on the activity of BAD_1528.
A, pH dependence of BAD_1528 activity in various buffers at 40 °C for 20 min. Enzyme activities are expressed as a percentage of the activity in acetate buffer at pH 5.0. B, Temperature dependence of BAD_1528 activity at pH 5.0 for 20 min. The enzymatic activities are expressed as the percentage of the activity at 40 °C. Buffers: sodium acetate (closed square) and MES (open circle).

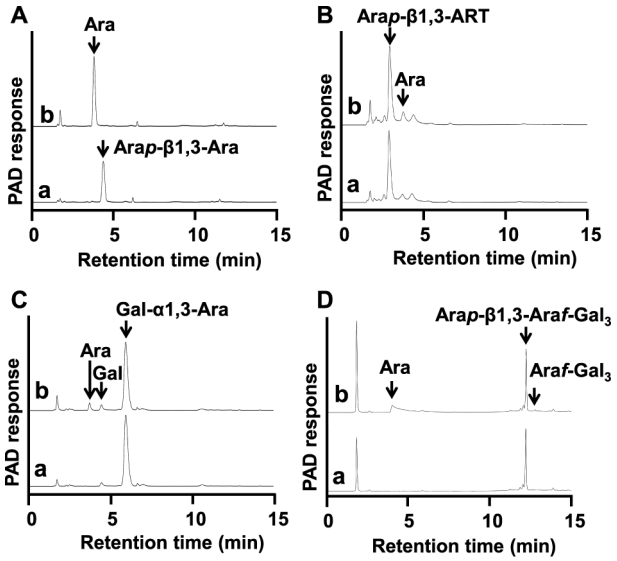

Fig. 5. HPAEC-PAD analysis of BAD_1528 reactions with disaccharides and oligosaccharide.
Ara*p*-β1,3-Ara (A), Ara*p*-β1,3-ART (B), Gal-α1,3-Ara (C), and Ara*p*-β1,3-Ara*f*-Gal$_3$ (D) were incubated with (b) or without (a) BAD_1528 at 40 °C for 16 h.

Table 1. Kinetic parameters of BAD_1528.

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
*p*NP-β-Ara*p*	1.48±0.04	81.5±0.51	54.9
Ara*p*-β1,3-Ara	8.69±1.9	7.66±1.6	0.881
*p*NP-α-Gal	5.96±0.03	2.43±0.01	0.408

form, Ara*p*-β1,3-Ara is thought to be present in the form of pyranose as Ara*p*-β1,3-Ara*p* in aqueous solution.

Table 2. Substrate specificities of BAD_1528.

Substrate[a]	Specific activity (Units/mg)	Relative activity[b] (%)
*p*NP-β-Ara*p*	46.8	2480
Ara*p*-β1,3-Ara[c]	1.89	100
Ara*p*-β-Ara*p*-β-*p*NP[d]	1.07	56.6
Ara*p*-β1,3-ART	0.00155	0.0820
Ara*p*-β1,3-Ara*f*-Gal_3	Trace	Trace
*p*NP-α-Gal	0.903	47.8
Gal-α1,3-Ara	0.00335	0.177

[a]The substrate concentrations of *p*NP-β-Ara*p*, Ara*p*-β1,3-Ara, Ara*p*-β-Ara*p*-β-*p*NP, Ara*p*-β1,3-ART, Ara*p*-β1,3-Ara*f*-Gal_3, *p*NP-α-Gal, and Gal-α1,3-Ara were 8.0, 5.0, 0.77, 0.25, 0.25, 8.0, and 0.44 mM, respectively. [b]Relative activity is expressed as the percentage of the activity toward Ara*p*-β1,3-Ara. [c]The specific activity was calculated on the value for released L-arabinose by one-half. [d]The specific activity was calculated on the value for released L-arabinose.

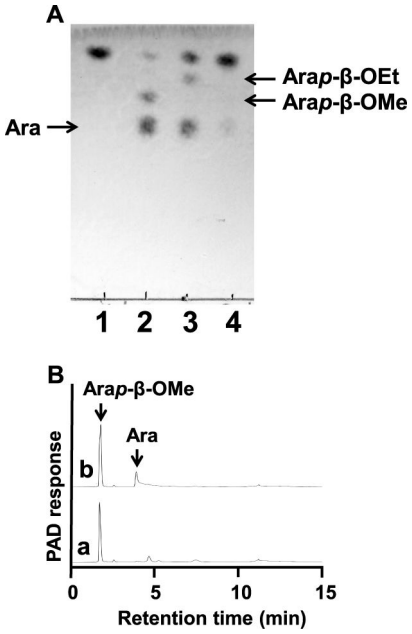

Fig. 6. Transglycosylation activity of BAD_1528 in the presence of 1-alkanols.

A, BAD_1528 was incubated with *p*NP-β-Ara*p* in the presence of either 10 % methanol (lane 2), ethanol (lane 3), or 1-propanol (lane 4) at 40 °C for 2 h. Lane 1, *p*NP-β-Ara*p* standard. Me, methyl; Et, ethyl. B, Purified Ara*p*-β-OMe was incubated with (b) or without (a) BAD_1528 at 40 °C for 16 h.

BAD_1528 is a novel exo-β-L-arabinopyranosidase that recognizes the Ara*p*-β1,3-Ara*p* structure. It is considered that Ara*p*-β1,3-Ara degradative enzymes may be present in almost all bifidobacterial species, because the homologous genes (59.3–81.3 % identities with BAD_1528) were conserved in several bifidobacterial species shown in Fig. 1B. Although Ara*p*-β1,3-Ara can be prepared artificially by partial acid hydrolysis, its existence in nature has never been reported. Recently, we have confirmed the Ara*p*-β1,3-Ara releasing activity in *B. longum* JCM7052 (unpublished data). To the best of our knowledge, AGPs are the sole source of Ara*p*-β1,3-Ara production. We also characterized an AGP degradative enzyme and predicted the AGP metabolic pathway in *B. longum*.[18] Interestingly, *B. longum*

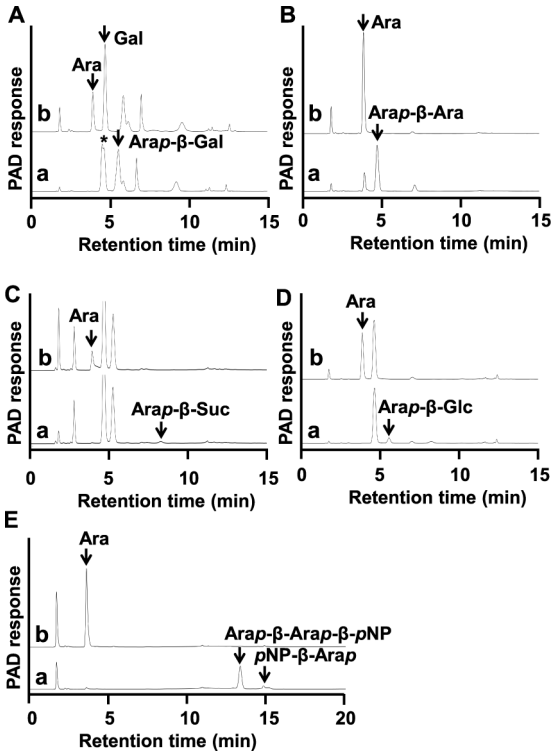

Fig. 7. HPAEC-PAD analysis of BAD_1528 reactions with transglycosylation products.

Ara*p*-β-Gal (A), Ara*p*-β-Ara (B), Ara*p*-β-Suc (C), Ara*p*-β-Glc (D), and Ara*p*-β-Ara*p*-β-*p*NP (E) were incubated with (b) or without (a) BAD_1528 at 40 °C for 16 h. A shoulder peak indicated by the asterisk was predicted to be Ara*p*-β-Ara.

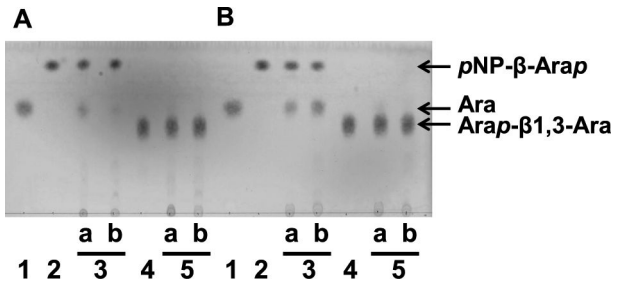

Fig. 8. TLC analysis of β-L-arabinopyranosidase activities in *B. adolescentis* strains.

The sonicated cell lysates of *B. adolescentis* JCM1275 (lane a) and JCM15918 (lane b) cultured in the MRS medium supplemented with glucose (A) or galactose (B) were incubated with *p*NP-β-Ara*p* (lane 3) or Ara*p*-β1,3-Ara (lane 5) at 40 °C for 16 h. Lane 1, L-arabinose standard; lane 2, *p*NP-β-Ara*p* standard; and lane 4, Ara*p*-β1,3-Ara standard.

JCM1217 exhibited no β-L-arabinopyranosidase activity, and GH27 β-L-arabinopyranosidase was predicted as a pseudogene without transcriptional control of its gene expression.[11] AGPs may be assimilated in cooperation with several bifidobacterial species through cross-feeding as an important carbohydrate source.

Several unique α-L-arabinofuranosidases have been reported in *B. adolescentis* for the degradation of arabinoxylan-oligosaccharides, such as GH43 arabinoxylan arabinofuranohydrolase AXHd3 (BAD_0301)[21][22] and GH51 α-L-arabinofuranosidase (BAD_1524),[22] which release only C3-linked arabinosyl residues from double-substituted xy-

lose residues, and AXHm23 (BAD_0423),[22] which releases arabinosyl residues from single-substituted xylose residues. Furthermore, it has GH1 α-L-arabinofuranosidase specific for α1,5-arabino-oligosaccharides (BAD_0156).[23] We also characterized neighboring genes of BAD_1528: BAD_1525 as GH27 α-D-galactopyranosidase/β-L-arabinopyranosidase, BAD_1527 as GH43 α-L-arabinofuranosidase, and BAD_1529 as GH127 β-L-arabinofuranosidase (unpublished data). The conservation of these L-arabinosidases in *B. adolescentis* is meaningful from the following viewpoints. L-Arabinose can be used by some bifidobacterial species as a carbohydrate source, although substrate preference varies by species. In this study, *B. adolescentis* strains did not grow on Ara*p*-β1,3-Ara as the carbohydrate source, in spite of the presence of intracellular β-L-arabinopyranosidase activity, which indicates that Ara*p*-β1,3-Ara is not good carbohydrate source for *B. adolescentis* similarly to L-arabinose. Pastell *et al.* reported that α-L-arabinofuranosidases are needed for the elimination of L-arabinose residues on xylooligosaccharides, and the releasing L-arabinose may be used by other bifidobacterial species such as *B. longum*.[24] As a result, various L-arabinosidases in *B. adolescentis* may play important roles in bifidobacterial symbiosis by providing L-arabinose that can be used as an energy source to other bifidobacterial species. In addition, these enzymes are predicted to be accessory enzymes for the intracellular degradation of plant oligosaccharides. There are several reports that α-L-arabinofuranosidases have synergistic effects with β-xylanases for the arabinoxylan degradation.[25][26][27] Therefore, releasing L-arabinose from oligosaccharides is important for facilitating access of other enzymes to the substrates.

In this study, we confirmed β-L-arabinopyranosidase activity in two strains of *B. adolescentis* (JCM1275 and JCM15918). *B. adolescentis* JCM1275 encodes three β-L-arabinopyranosidase candidates, i.e., one GH27 (BAD_1525) and two GH36s (BAD_1528 and BAD_1576), whereas JCM15918 encodes only two GH36s (BSTER_1815 and BSTER_1892). Because β-L-arabinopyranosidase activities were detected in both JCM1275 and JCM15918, GH36 enzymes may be responsible for the β-L-arabinopyranosidase activity. However, it is not certain whether or not BAD_1528 and the ortholog BSTER_1892 are expressed in each strain. BAD_1576 or the homologous enzymes encoding α-D-galactopyranosidases have the potential to have the β-L-arabinopyranosidase activity, although it has not been reported yet.[14][15][16][17] Therefore, additional functional characterization and transcriptional analysis of BAD_1528 and BAD_1576 may be necessary.

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